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Quantitative Methods in Histology and Microscopic Histochemistry

WITHDRAWN

By

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PREFACE

> "I often say that when you can measure what you are speaking about and express it in numbers you know something about it; but when you cannot express it in numbers, your knowledge is of meager and unsatisfactory kind: it may be the beginning of knowledge, but you have scarcely, in your thoughts, advanced to the stage of *science*."
>
> LORD KELVIN: Popular Lectures and Addresses. 1883.

Consultation of recent histological and histochemical literature shows that the microscopical specimens are still mostly described in purely qualitative terms. This is undoubtedly due mainly to the descriptive nature of these branches of biology, and in many cases there is no necessity of using more objective and accurate terms. However, there are also many problems in histology and histochemistry which are difficult to study without a quantitative approach. This is particularly true in experimental studies, where many minor changes may be overlooked in purely qualitative examination or in which subjective bias may badly distort the results.

At present, no systematic introduction to the quantitative methods in histology and microscopic histochemistry is available, although much valuable information is scattered in recent literature. This little book is a humble effort to fill the need of such a guide. It is hoped that it will prove useful in the hands of histologists and histochemists who are not familiar with the possibilities for quantitative work in their field and also to the students of various branches of experimental biology who would welcome the addition of quantitative microscopical methods to their armament.

It must be made quite clear that only methods with which the writer has had at least some personal experience are discussed and that some important quantitative techniques are therefore excluded. No priority is claimed over the methods presented, many of which have long been in use, if not in histology or histochemistry, perhaps in geography or chemistry.

As basic knowledge of statistics is essential in all kinds of quantitative work, I am very glad to have been able to persuade Mr.

Jaakko Kihlberg to collaborate in the writing of this volume. He is fully responsible for chapters I and XI, and he has essentially contributed to chapters VI and VII. It has been a pleasure to work with him.

The idea of compiling this volume ripened in the course of the academic year I spent in the University of Edinburgh, and a considerable proportion of actual writing was done during that period. I am very grateful to Professor *J. H. Gaddum* for the time I had the opportunity of spending in his department, and to Dr. *M. Vogt*, under whose guidance I had the pleasure of working. I also owe my best thanks to Dr. *R. Barer*, of the University of Oxford, who kindly demonstrated his ingenious methods and equipment during my visit to Oxford. He has further given me valuable advice both orally and in numerous letters as well as helped in the construction of a photometer. I am much indebted to many other British colleagues, particularly to Dr. *A. G. E. Pearse*, of the Postgraduate Medical School of London University, as well as to Dr. *K. W. Metcalf*, of the University of Bristol, and to Drs. *F. Jacoby* and *B. F. Martin*, of the University of Cardiff, for stimulating discussions on histological and histochemical problems related to the subject of this volume. My journey to Great Britain was made possible by a grant given by the *British Council*, which is gratefully acknowledged. Dr. *M. J. Karvonen* has given valuable suggestions and assisted in the proof-reading, for which I am very grateful to him. Thanks are due to Dr. *H. R. Mitchell* for correcting the English and to Mrs. *T. Rytilä* for typing the manuscript.

I should also like to thank Dr. *R. C. Mellors* and *the American Association for the Advancement of Science* for permission to reproduce Fig. 21, Messrs. *Kodak Ltd.*, London, for Figs. 35 and 36, and Messrs. *Mullard Ltd.*, London, for Fig. 42. A number of figures from various sources have been reproduced slightly modified; the corresponding references are given in the text.

For excellent co-operation and friendly dealings I wish to thank Dr. *H. Karger* and Messrs. *S. Karger AG.*, Medical Publishers.

Autumn 1954. O. E.

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INTRODUCTION

All histological and a great proportion of histochemical observations are done by examining tissues under the microscope. To illustrate the great importance of subjective judgment in this respect, let us consider a blood smear under a high magnification. A haematologist might describe the view shortly as follows: «There are strongly coloured, large erythrocytes, some of which are oval. Probably pernicious anaemia.»

Let us then show the same visual field to a layman, say to an artist, who can be expected to have experience in description of forms and colours. The artist might think that the corpuscles are pale, small rather than large, and remarkably circular, i.e. his opinion would be the exact opposite to that of the haematologist.

Both opinions can be motivated. The haematologist has seen thousands of blood smears and the cells now concerned seem to him large, strongly coloured and aspherical in comparison to his idea of normal erythrocytes. The artist, on the other hand, compares the colour of the erythrocytes to the strong red hues used by expressionists, and the monotonous sphericity of these cells to the rich forms of non-figurative sculptures. That the cells are small is obvious to him because a microscope is necessary to make them visible.

Although "large" to a haematologist is something else than "large" to an artist, his description may nevertheless convey the right meaning to another haematologist, whose subjective criteria are largely similar. However, even every haematologist is different from any other haematologist as is every histologist from any other histologist, and discrepancies may appear. These discrepancies can be eliminated by using objective instead of subjective criteria, i.e. by measuring instead of assessing.

The use of quantitative instead of qualitative terms is always combined with extra trouble. If one wants to answer the question "how large?" instead of just saying "large", measurements must be done. However, measuring every erythrocyte in the blood of a man would keep the whole population of London busy 8 hours a day for six months, supposing that 1 second is given for measuring each cell. This creates a new question "how many cells are to be measured?" Suppose that this problem is solved and the measurements are carried out. A number of figures is obtained and with them new questions: "what to do with these figures?", "how to compare this data with that obtained from another person?", etc.

To solve these questions, the investigator must be familiar with statistical principles. Though there still are scientists who think that statistics is a kind of scientific snobbery by which right things are proved wrong and vice versa, an effort to understand the statistical method will prove to anybody the value and necessity of statistical considerations in design of experiments and in interpretation of quantitative data.

In many biological papers either inaccuracy of the method or the limited number of observations has been said to prevent the use of statistical methods. However, the main basis of statistics is the necessity of using quantitative methods which are not faultless, and of limiting the number of observations. Results obtained with a small material particularly need statistical analysis and may then be found sufficiently reliable. Creation of pertinent testing methods for small samples is indeed one of the most important achievements of modern statistics.

It has frequently been claimed that, e.g., absorption measurements on stained tissue sections are useless unless it can be shown that reliable information is thus obtained regarding absolute amounts of some specific substances in the tissue. This is equivalent to throwing the child away with the washing. A change in the absorption properties of a stained tissue may, it is true, be due to increase, decrease, or redistribution of stainable materials, to loss or gain in the stainability of these materials, to appearance of new materials reacting with the dye, to an altered physical state or indeed nearly anything else. Still, if the change in the absorption has been proved to depend on some experimental procedure it is completely justifiable to use absorption measurements as an empirical means of obtaining information of the state of the tissue. Generally, any quantitative method can be used, although many variables would simultaneously influence the property to be measured.

This volume describes means by which figures can be extracted from tissue sections and tries to show the critical ways of handling the figures thus obtained. No effort has been made to include all quantitative methods hitherto published. Many valuable techniques, e.g. polarization microscopy, have not been discussed at all. The two aspects mainly concerned are the quantitative topography of and light absorption measurements in tissue sections. Even these subjects are broadly outlined and the emphasis has rather been on the presentation of basic facts in an understandable way than in thorough review of the knowledge accumulated. The result is a mixture of histology, physics, chemistry, and statistics. It has been tried to make the mixture palatable, by using simple words and no mathematics above the high-school level; and nourishing, by combining materials which complete each other's value. Key references are given to modern literature, frequently without regard to historical or priority aspects.

Many methods of quantitative histology and histochemistry require complicated equipment both expensive and difficult to use. As such apparatus can anyway be reached by a few laboratories only, and as the writer's experience with them is negligible or zero, they are not dealt with in detail. Practically all methods to be presented are possible with equipment either already available in most histological laboratories or easy to construct without excessive funds or technical facilities. It is felt that there is an unnecessarily big gap between highly specialized quantitative studies done with complicated and ingenious devices and studies in which no attempt has been done to quantitate the data. Obviously, as has frequently been pointed out by masters of the advanced methods of quantitative histochemistry, there are many sources of error in the quantitative methods. This fact does nevertheless not eliminate the use of simple techniques as long as their limitations are kept in mind and the results obtained with them are correctly interpreted. This volume tries to point out the numerous sources of error and give hints to their control. Basic knowledge of the various aspects of quantitative methods is believed to be useful not only to the histologist doing quantitative work in practice but also to his colleague who does not like bias although he prefers to express his observations in qualitative terms.

CHAPTER I

Variability and its Measures

Distributions.

Let us first return to the example of red blood cells mentioned in the introduction. Suppose that the size of the erythrocytes is under investigation. The investigator may have measured diameters of 423 erythrocytes. This series of numbers may be further studied in different ways. One way, which has proved to be of great value, is to put the figures into the order of magnitude. The number of erythrocytes of each size is then counted (this were naturally impossible, if the diameters had been measured so precisely that all cells had different diameters; here we suppose that the diameters have been measured to the nearest half of a μ). The number of cells of some size x is then the *frequency* n_x of this size. By putting the diameters on the abscissa and the corresponding frequencies on the ordinate a *frequency distribution curve* is obtained. The frequency can as well be expressed as per cent of the total number of ob-

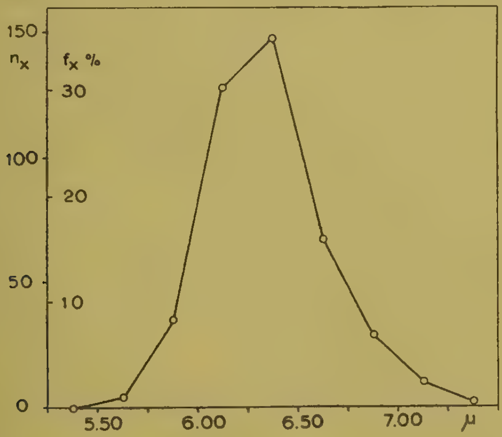


Fig. 1. Diameter-frequency distribution curve of red blood cells.

servations (relative frequency f_x) which in the present example is simply done by dividing the absolute frequency by 4.23. A curve like this is well known amongst clinicians as a Price-Jones curve. An actual curve is illustrated in Fig. 1.

The distribution of some other property of the erythrocyte, such as the haemoglobin content, can be examined in the same way. In general, the empirical distribution of any observed variable is similarly obtained.

In many applications it is useful to form a series of *cumulative* frequencies by adding step by step the frequencies upwards from down (i.e. from the smallest size-class) (Fig. 2). As the frequency f_x tells how great a proportion of the observations belong to a given size-class, the cumulative frequency F_x tells how great a proportion belongs to this class or to classes of smaller size.

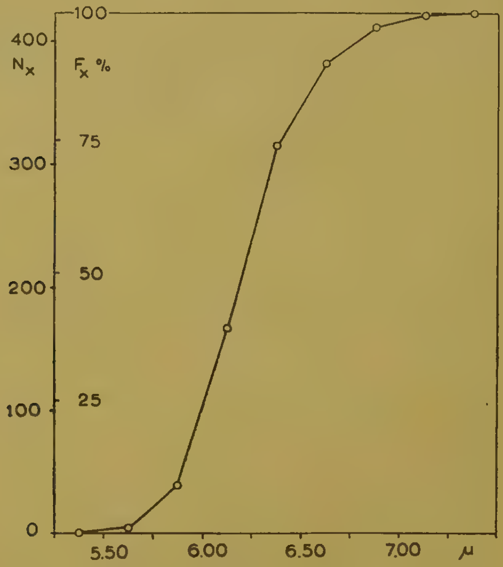


Fig. 2. Cumulative distribution curve prepared from the same data as Fig. 1.

In grouping observations the number of classes should not be made unduly large. A practical rule is that the number of classes should be approximately equal to the cube root of the number of observations. For example, some 30 observations should be grouped in 3 or 4 classes, some 100 observations in 4 or 5 classes, 1000 observations in about 10 classes and so on. Generally, the number of observations in a single class should not remain smaller than 5.

If the distribution is skew, i.e. if the frequency curve shows a "tail" in one or another direction, it may be useful to apply unequal class intervals, whereas the intervals should be kept constant in all ordinary classes. In particular, if the logarithms of the observed values are of interest (see page 15), the class boundaries can be chosen in geometric progression, for example forming the classes (in arbitrary units) 1, 2 . . . 3, 4 . . . 7, 8 . . . 15, 16 . . . 31, etc., in this case the lower boundary of the class rising in geometric progression. Note well that a) the cumulative frequency curve is unaffected by the grouping, but b) the frequency curve must be adjusted for unequal class intervals. For example, if the per cent frequencies are

25 % in size class 2 ... 3
15 % in size class 4 ... 7,

the interval is two units in the first class, the frequency being thus $\frac{25}{2} = 12.5\%$ *per unit*, whereas the interval in the second class is 4 units, the frequency per unit being $\frac{15}{4} = 3.75\%$. The frequency curves should always be drawn adjusted as described here.

Two main types of distributions can be distinguished on the basis of the nature of the variable in question, viz. a) continuous and b) discrete distributions. All size distributions, for example, are continuous, as the variable (the size) can obtain any value on the continuous size axis. All distributions based on counting, on the other hand, are discrete, as the result of counting can obtain integral values only.

Special Distributions.

In building mathematical models so as to aid the treatment of statistical data, the *distribution functions* play an important role. A distribution is said to be completely known if there is a mathematical equation with one or more *parameters* by which one can calculate the frequencies. A great number of such distribution functions are known, and some of them will be presented here.

Certainly the most important distribution is the Gaussian or the *normal distribution.* The shape of the normal frequency curve is shown in Fig. 3. The curve is symmetrical around its *mean* and decreases rather rapidly towards both tails. Besides the mean the distribution function has another parameter, viz. the *standard deviation* σ, estimated by s, the square root of the variance estimate s^2

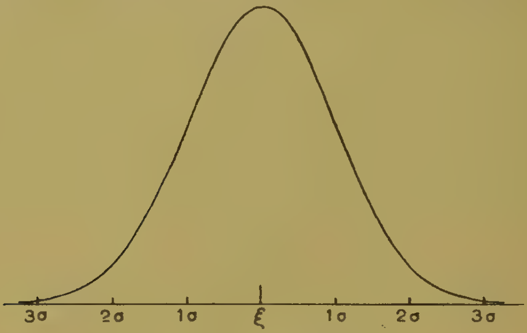


Fig. 3. Normal distribution.

based on the material (see page 20). The standard deviation measures the dispersion of the distribution in such a manner that approximately

a) 68 per cent of the normal observations will lie within a region mean ± standard deviation,

b) 95.5 per cent of observations will lie within mean ± twice the standard deviation,

c) 99.7 per cent will lie within mean ± three times the standard deviation.

The practical importance of the normal distribution is in that

a) many actual observations tend to be distributed closely according to the normal law,

b) certain statistical characteristics, notably the mean, tend to be normally distributed even if calculated from a non-normal material.

Often not the observed variable itself but rather some *transformation* of it is normally distributed. In many biological applications, the *logarithm* of the variable is distributed closely to the normal law. If so, the distribution is called *logarithmico-normal*, or briefly log-normal. The usefulness of logarithmic transformation is illustrated by the fact that when any variable is log-normally distributed the squares and cubes of this variable also show a log-normal distribution. For instance, when nuclear diameters are log-normally distributed the volumes are also so.

When the distribution of the mean is concerned, it is customary to change the term standard deviation into *standard error*, so that standard deviation is a measure of dispersion of the *observations*, standard error being a measure of dispersion of statistical *characteristics* such as the mean, the correlation coefficient, etc. It should be noted that this distinction is a matter of language only, for theoretically the two terms are identical.

Another important distribution is the *binomial distribution*. If n points are thrown on a section where the proportion of some specific surface is P, the number m of hits on the specific surface is a binomial variable with parameter P (this distribution has only one parameter). The expected value or mean of m is Pn, and its standard deviation is the square root of $P(1 - P)n$. If P is not nearly $\frac{1}{2}$ and if n is small, the binomial frequency curve is skew, but P being near by $\frac{1}{2}$ and n growing large, the curve becomes symmetrical and resembles the normal curve. Note, however, that the binomial variate is discrete, as m cannot obtain other than integral values.

Related to binomial is the *Poisson distribution*, which sometimes is called the law of small numbers. If particles on a tissue section are distributed with uniform probability over the area, and if small squares or circles are taken and the number x of particles in each square or circle counted, the variable x will have the Poisson distribution. The mean or expected value of x is m, the parameter of the distribution, and the standard deviation of x is the square root of m. If m is small (m being the average number of particles per square or circle), the frequency curve is skewed, but becomes symmetrical and resembling the normal curve if m is increased. Like the binomial this distribution is discontinuous.

It should be noted that the presumption on the pattern of particles on the tissue given above does not hold universally. On the contrary, often there are apparent reasons to expect non-Poisson distribution because the particles appear in clusters. Therefore, it is advisable to test whether the Poisson assumption actually holds.

Sampling Variability.

Above it was stated that the distributions of certain statistical characteristics tend to the normal distribution. This involves the general feature that any statistical characteristics, say the mean, if calculated from a limited material, a *sample*, will show some variability from one sample to another. Often the other samples are hypothetical (say from the ideal population of all possible experiments), but the sampling variability yet must be taken into account. But this variability follows certain mathematical laws which could be empirically verified if new samples were repeatedly taken. The characteristics calculated from a sample have thus their *sampling distribution:* for example the sample mean will fluctuate around the true mean with approximately normal distribution. Many more distributions of sample characteristics tend to the normal.

Therefore, there is certain distinction between the *distribution of the observations* themselves and the *sampling distributions of characteristics* based on samples. The same mathematical formulas may be applicable in both cases, but in most practical situations the number of observations in the sample enters the mathematical formulation of sampling distributions. For example, if in the normal distribution the true mean is $\bar{x}'$ and the true standard deviation s', then the sample mean $\bar{x}$ from a sample of n observations will vary around the true mean, again with a normal distribution with mean $\bar{x}'$ but with standard deviation $\frac{s'}{\sqrt{n}}$, which quantity now is called the *standard error* of the mean. Similar situations occur in connection with other characteristics also, e.g. per cent of hits, average count, etc. Note especially that the sampling distribution is changed if the number of observations changes. In particular, the precision of the characteristics increases if the number of observations increases, for in most practical cases the standard error of the characteristic (being a measure of the precision) is inversely proportional to the square root of the number of observations.

Randomization and Sampling.

As all natural phenomena are subject to variation, the interpretation of quantitative data is always somewhat uncertain. In designing experiments, one tries to keep under control or constant as many factors as possible, so as to ensure some safety in the conclusions. However, it is not always known in advance which really are the relevant factors. Therefore the risk is involved in any design that some important factor has been overlooked. To reduce the effect of this risk, *randomization* of the experimental conditions is the best device known so far. Suitably chosen selection methods should always be applied both in the general set-up of the experiment and in sampling.

Generally, *pure randomization* simply means a lottery-gamble between the individuals in the population to be studied, so arranged that every individual in the population has an equal chance to "win", i.e. to be selected in the sample. For example, if ten animals are to be selected from a group of 100, every animal must have one chance in ten to be selected. Or, if a square or a circle is to be chosen under the microscope to *represent* all the vision field, every location of this square or circle within the vision field must be equally probable (note that in this latter case the "population" to be sampled, the vision field, is no more composed of a number of separable "individuals" but is a continuous area). The best method to perform the lottery-gamble is to use random numbers which have been published in several collections of statistical tables (e.g. *Hald*, 1952; *Fisher* and *Yates*, 1953). These numbers have been especially produced in order to guarantee a perfect random order, and their mathematical nature in this sense has been carefully tested. The use of these tables is better than to use gambling with dice, cards or coins, because these may produce biased results although the investigator may not be aware of it.

Instead of pure randomization, *systematic selection* is often applied. This means that the individuals are chosen with fixed intervals, e.g. taking every other or every tenth animal out of one hundred. Similarly, a systematic sample from an area will be composed of equally-spaced points, lines or squares, etc. In microscopic work, the systematic sample generally is more practical than purely random one, wherefore further sampling questions in the subsequent sections will be treated also in view of systematic sampling. The random element in systematic sampling is introduced by "random start": if ten animals are systematically selected from one hundred, every tenth is taken, but the first animal must be determined at random, i.e. by gambling between the first ten animals.

Measures of Central Tendency.

In expressing the average observation, the *arithmetic mean*

$$\bar{x} = \frac{\sum x}{n} \tag{1}$$

is commonly used, where $\sum x$ is the sum of all observations, the number of the latter being n. It should be noted, however, that in skew distributions this mean is not always preferable because it is sensitive to great deviations from the center. In fact, in very skew cases, this mean may be quite far away from the "typical" or most common values of the variable. Consequently, in such cases other measures of central tendency should be used.

In certain calculations *weighted means* are needed. For example, if proportional area measurements p_i have been obtained for sections of different sizes a_i, a plausible over-all average proportional area is to be calculated as

$$p = \frac{\sum a_i p_i}{\sum a_i}, \tag{2}$$

i.e. the separate measurements are to be multiplied by the "weights" (here: areas), then summed, and this sum is to be divided by the sum of the weights.

Formula (2) is general in the sense that no presumptions are involved with regard to the weights a_i, i.e. they can be chosen at will.

The usefulness of the arithmetic mean is, besides in the simplicity of the calculation, in certain mathematical properties of it. But above it was noted that the numerical value of this characteristic may be very misleading in skew distributions. Therefore, other measures are needed. One in common use is the *median*, viz. the value of the variable which divides the material in two: one half being at most of the size of the median, the other half being larger (e.g. LD 50 as used in bioassays). Numerical estimate for the median can be quickly obtained from a cumulative distribution curve. Median is not sensitive to extreme deviations like the arithmetic mean.

In some applications the variate values are replaced by some *transformations*, e.g. logarithms. Then, the average transformed value can be obtained. Now it should be noted that for example the antilogarithm of the mean of the logarithms is *not* equal to the arithmetic mean (but is the *geometric mean*), and similarly for any transformations.

Measures of Variability.

Above the standard deviation was introduced and it was shown how this quantity measures the variability of the material. The square of the standard deviation is known as the *variance*, and as variance rather than standard deviation is simpler to use in certain calculations, the variance will subsequently be used. Its relation to the variability of the material can be derived by the following considerations. All the variability of the material can be expressed in terms of deviations from the mean, $x - \bar{x}$, or rather (by certain analogies in Newton mechanics) in terms of the squares of these deviations, $(x - \bar{x})^2$. A general definition of the variance is, then:

$$\text{Variance} = \text{Expected (or average) value of } (x - \bar{x})^2.$$

In dealing with n observations x, the first step is to calculate the *sum of squares*

$$Q_x = \sum (x - \bar{x})^2 = \sum x^2 - \frac{(\sum x)^2}{n} \tag{3}$$

the latter form being suitable for practical computations. The term $\frac{(\sum x)^2}{n}$ is known as the *subtraction term.*

Then, the variance of x is estimated by

$$s^2(x) = \frac{Q_x}{n-1}. \tag{4}$$

Here, $n - 1$ is the number of *degrees of freedom*. Generally, any sum of squares divided by the number of degrees of freedom corresponding to it gives a variance estimate.

Some formulas will be useful in the subsequent. First of all, the *variance of the mean* of n observations is estimated by

$$s^2(\bar{x}) = \frac{s^2(x)}{n}. \tag{5}$$

In binomial case, the estimation formula for the variance of the number of hits, m, is

$$s^2(m) = \frac{n^2 p(1-p)}{n-1}, \tag{6}$$

where p is the observed frequency of hits, $p = \frac{m}{n}$. Further, the corresponding formula for the variance of p is

$$s^2(p) = \frac{p(1-p)}{n-1}. \tag{7}$$

In some presentations, n instead of $n - 1$ is used in (6) and (7). However, that procedure is biased if p in the formula is not the true value P. In practice, it usually is the actually observed proportion in the sample, i.e. an estimate of the true value, which leads to the conclusion that the number of degrees of freedom $n - 1$ must be used instead of n.

In Poisson case, the variance estimate equals to the mean count per counting area. If the individual counts are denoted by x and there are n of them, we have

$$s^2(x) = \bar{x} = \frac{\sum x}{n}. \tag{8}$$

The sampling variance of the mean count $\bar{x}$ is then

$$s^2(\bar{x}) = \frac{s^2(x)}{n} = \frac{\bar{x}}{n}. \tag{9}$$

Two general variance formulas should be noted as they will be useful in subsequent applications. First, if the statistical variable is multiplied by a constant, then the variance will be multiplied by the square of the constant, thus:

$$s^2(cx) = c^2 s^2(x)\,. \tag{10}$$

Note that the formula involves also the case that x is divided by a constant.

Secondly, the variances of *independent* variables are additive so that the variance of the sum of two *independent* variables x and y is the sum of their variances:

$$s^2(x + y) = s^2(x) + s^2(y)\,. \tag{11}$$

Formula (11) also gives the variance of the difference $x - y$. To be accurate, a necessary and sufficient condition for (11) is that x and y have a *correlation of zero* (see page 25). If the correlation coefficient r_{xy} is not equal to zero, an additional term $2r_{xy}s(x)s(y)$ is introduced.

Sometimes we are interested in the *relative variability* of the observations rather than in the absolute values. If so, it is customary to calculate the *coefficient of variation*, which is defined as the standard deviation (error) divided by the mean, thus:

$$V(x) = \frac{s(x)}{\bar{x}}\,. \tag{12}$$

This quantity is often expressed in percentage, thus giving a quick idea of the relative importance of the variability in the material.

Use of Standard Deviation and Standard Error.

In practice the standard deviation and standard error are used as indicators of the variability of the material or the precision of the sample estimates (characteristics). The variance was defined with no respect to the form of the distribution so that the use of the standard deviation or error does not involve any assumptions concerning the distribution. However, in most practical situations and frequently in the literature the normal distribution is held in mind when presenting standard deviations or errors.

For example, the sample mean $\bar{x}$ is an estimate of the true mean (which is unknown), and it may or may not equal that. Now the standard error $s(\bar{x})$ estimates the variability of the sample mean around the true mean in such a way that approximately the rules on page 14 hold, giving thus an idea of variability of the sample mean. Deviations larger than twice the standard error from the true mean are likely to occur 4-5 times in 100. From this it is concluded that the sample mean is not likely to be more far away from the true mean than twice $s(\bar{x})$, or, for example, practically certainly not more far away than three times $s(\bar{x})$. Therefore, the standard error can be used in constructing what are known as *confidence belts*. As the true mean is unknown, the observed sample mean must be replaced for it, so that for example the belt $\bar{x} \pm 2s(\bar{x})$ is to be interpreted as follows: in approximately 4-5 cases out of 100 this interval does not include the true mean, i.e. there is about 95.5% confidence in that this interval includes the true mean.

The principle "twice the standard error" is an old practice and we have used it for illustration only. There is a bias involved in that the true standard error is unknown and replaced by the estimate $s(\bar{x})$. This bias is corrected by the use of the t-distribution (see page 135) instead of normal. From the t-distribution (tabulated in several collections of statistical tables) the coefficients to be replaced for 2 (or 3) above can be found for any number of observations and for any desired confidence level. The confidence level, 95.5% in the example above, can thus be chosen at will. Usually conventional levels like 95 or 99 or 99.9 per cent are used.

Now this procedure is approximately correct only if the sampling distribution of the mean is normal. In most practical cases it is approximately so if the sample is large enough, independent of the form of the distribution of observations themselves, but always caution is needed. In particular, if the distribution in question is skew instead of normal and symmetrical, the procedure above may lead to dangerously misleading results. For example, the sampling distribution of the correlation coefficient r is far from normal if the true correlation deviates from zero. Hence, it is not advisable and generally not correct to use standard error in connection with correlation coefficient if the true coefficient cannot be taken equal to zero (see page 137).

Determination of the Error of Method.

The term *error of method* refers to sources of error other than sampling, e.g. to inaccuracies in the drawing or measurement of particles or cells.

Sometimes, especially in extensive studies, it is necessary to estimate the magnitude of the effect of this source of error. Commonly, duplicate measurements are made in this purpose, the determination of the method error then being based on comparisons between the duplicates:

x_1	x_2	1st measurement duplicated
x_1	x_2	2nd measurement duplicated
...	...	...
x_1	x_2	*n*th measurement duplicated

There are n pairs of measurements. Denote d for $x_1 - x_2$, the difference between the duplicates in each case, so that n figures d are to be calculated. Then

$$s^2 = \frac{\sum d^2}{2n} \tag{13}$$

is a simple and quickly calculated formula for the *error variance*, the square root of which is the *standard deviation of a single measurement*. This quantity is usually given as the *method error*.

Earlier (see page 20) it was stated that any sum of squares divided by the number of degrees of freedom gives a variance. It should be therefore noted that the number of degrees of freedom in the formula is *not* $2n$ (neither $n - 1$) but n, the sum of squares actually in question being $\frac{\sum d^2}{2}$. This can be verified if the procedure is looked from the angle of view of the analysis of variance (see page 138). What has been made is that each pair is considered as a "group", whereafter the between groups variation has been eliminated. The sum of squares $\frac{\sum d^2}{2}$ represents the variation within groups, being thus purified from the effect that the n measurements may be unequal (e.g. sections of different size).

When n is large, an accurate estimate of the method error can generally be obtained. However, if n is small or if great accuracy is required but it is not possible to increase n, then triplicates, quadruplicates, etc. can be recorded. As *Fisher* (1950) points out, this procedure may even be essentially more effective than to increase n. In case of triplicates, the series has the form

x_1 x_2 x_3 1st measurement triplicated
x_1 x_2 x_3 2nd measurement triplicated
... ...
x_1 x_2 x_3 nth measurement triplicated

In order to obtain the method error, consider the n measurements as n "groups", and proceed then exactly in line with common analysis of variance (see page 138). Calculate the total sum of squares, reduce from it the sum of squares between groups, and what remains is the sum of squares within groups, Q_o. This divided by $2n$ gives the error variance.

Similarly, if each measurement is reduplicated four times, the procedure is as above. Now the sum of squares within groups is to be divided by $3n$.

Interrelations Between Two Variables.

Numerical dependence exists between two variables x and y if any change in x corresponds to a change in y, either so that increasing x means increasing y or on the contrary. This dependence is called *correlation.* A measure of the degree of correlation is obtained by defining the *covariance:*

$$\text{Covariance} = \text{Expected (or average) value of } (x - \bar{x})\,(y - \bar{y}).$$

Note certain analogy with the definition of variance on page 19.

In mathematical statistics it is shown that this quantity equals to zero whenever x and y are numerically independent, increasing (in absolute values, for it can be also negative) if dependence exists. An estimate of the covariance is obtained by calculating first the *product-sum*

$$T_{xy} = \sum (x - \bar{x})(y - \bar{y}) \tag{14}$$
$$= \sum xy - \frac{(\sum x)(\sum y)}{n},$$

the latter form being suitable for practical computations. Note certain analogy with (3).

Then, the covariance is estimated by

$$Cov(xy) = \frac{T_{xy}}{n-1}. \tag{15}$$

Note certain analogy with (4).

Now the quantity

$$r_{xy} = \frac{Cov(xy)}{s(x)\,s(y)} \tag{16}$$

is known as the *correlation coefficient.* In case of complete independence, the coefficient equals to zero. If there is complete correlation (positive or negative), i.e. if the value of x exactly defines the value of y, r equals to $+1$ or -1.

To be accurate, the terms dependence/independence above refer only to *linear* dependence/independence. For example, if $y = x^3$, there is an exactly defined dependence between x and y, but r in this case is *not* equal to unity. Linear dependence exists in cases where a statistical variable y (the volume of a gland, say) can be satisfactorily estimated on the basis of another variable x (weight of the gland) by means of a linear regression equation (see page 143):

$$y \simeq a + bx.$$

It follows that an asymmetry holds: if x and y are independent, their correlation coefficient will be zero, but if the correlation coefficient is zero, it does not necessarily mean that x and y are independent (as they may be dependent in some non-linear form). Further: an observed correlation does not involve material dependence between x and y, but they between themselves may be independent, both, however, being correlated with a third variate.

CHAPTER II

Animal Experiments

Introduction.

Before the microscopic slide is ready for actual quantitative study, several steps must be taken. In a typical experiment, the following stages are to be passed: selection of experimental and control animals, experimental treatment, killing, taking a piece of tissue, fixation, embedding, cutting, floating on slide, staining. The properties of the set of sections thus prepared are essentially dependent on every stage of the whole procedure. Therefore, it is necessary to try to limit the influence of all non-specific factors to the minimum during every step of the work. Moreover, to be able to use statistical methods in the evaluation of the results, certain conditions outlined in the preceding chapter must also be kept constantly in mind.

Selection of Animals.

Completely misleading results are easily obtained unless correct methods are used in the selection of animals. This is the first step in each experiment where *sampling* is done and all sources of error combined with sampling are at hand (see page 17). If any space is left to subjective selection, a bias is likely to influence the final result although the rest of the investigation would be carried out faultlessly.

If the animals are taken out of a big cage, one at a time, those easier to catch will be taken first and those least interested in leaving the cage last. Although the interest of the animals in this respect may seem to matter little, it is certain that the first animals will differ from the last ones in more than one respect. Selecting the first animals to the control group and the last ones to the experimental group is biased and may cause either falsely positive or falsely negative results.

Taking every other animal to the control group and the rest to the experimental group is correct if the first is randomly selected. However, a subjective bias may be introduced. The investigator may be influenced by his expectations and unconsciously select, say, more vigorous animals to the experimental group. There are several examples of the real occurrence of this kind of bias even amongst experienced investigators.

The best way of preparing a perfectly randomized sample is to give a number to each animal and to use a table of random digits (see page 17). If three independent groups each of 10 animals are wanted, the first 10 animals are given by the first ten numbers in the table. The following series of numbers are taken from a table of random digits: (30, 28, 27, 25, 14, 7, 16, 15, 8, 6), (19, 12, 5, 3, 20, 1, 11, 18, 26, 10), (17, 4, 23, 29, 2, 21, 9, 13, 22, 24). With the aid of these numbers 30 animals can be safely divided at random into three experimental groups. Against what might be thought, it is not satisfactory to improvise a self-made series of numbers because this will not fulfil the conditions of randomization.

Sometimes the sensitivity of the experiment can be increased by using pairs matched, e.g., according to the body weight. One animal in each pair is then marked with a spot of ink and coin is tossed for each pair to decide whether the marked or unmarked animal should be put to the control group. The coin must of course be unbiased. Tossing a coin can also be used to select animals to three or more groups. For three groups, e.g., the following method may be applied, tossing the coin twice for each choice: head-head, group 1; tail-tail, group 2; head-tail, group 3; tail-head, repeat.

Randomization does in no way increase the sensitivity of the experiment, it only makes it unbiased. Matching, mentioned above, in which each experimental animal has a control of its own, may nevertheless greatly increase the sensitivity of the test by diminishing the effect of variation between animals. This is particularly the case when litter-mate controls are used. Many characteristics showing a high variability between animals randomly selected from the same inbred strain are surprisingly constant in litter-mates.

Number of Animals Needed.

How many animals are to be taken to each group is a problem which must be solved before the actual experiment is started. The optimum size of the group is very difficult to assess, even if details of the experimental conditions are known and earlier experiments give an idea of the probability that the working hypothesis is true. One is, of course, always on the safer side by working with large groups but this may be either impossible or unduly costly. Understanding of the statistical principles presented in the first and in the last chapter of this volume is nevertheless of considerable aid in making the decision. Here an example is given to illustrate the point.

Consider an experiment designed to make clear whether a given dose of nicotine influences the size of the adrenal medulla. Two groups of animals are taken, and one of them is injected with nicotine. The medullary volume is measured in each animal with the most accurate method, i.e. by planimetric measurements in a complete series of sections. Suppose then that in an experiment with 5 + 5 animals the means and the corresponding variances are 1.7 cu.mm. and 0.3 cu.mm. for the control group and 2.5 and 0.5 cu.mm. for the injected group, respectively. If the experimental conditions have been strictly randomized, the use of the *t*-test (see page 135) is justified. It will show that the observed difference in the medullary size is due to chance in about 1 case out of 10. This makes the hypothesis that nicotine does increase the size of the medulla rather doubtful.

Suppose now that the number of animals in both groups is increased to 10 and that the means and the variances will turn out to be the same as in the preceding experiment. Then the *t*-test gives the result that the hypothesis may be wrong in only 2 cases out of 100, which might be interpreted to mean that the nicotine treatment really has an effect.

These hypothetical experiments seemingly show that doubling the number of animals will radically improve the significance of the results. However, this should not be interpreted as to mean that large samples always are preferable. The point is that large samples enable the investigator to detect *smaller* differences than small samples. If the true effect of the treatment is negligible or zero, there is no sense in enlarging the sample, whereas, if the true effect is considerable, even small samples will detect the effect. Actually, the statistical methods are specifically designed to obtain a reasonable estimate of the influence of the random variability on the result, no matter what the size of the sample. Therefore, if the probability of the observed changes being due to chance is estimated at, say, 1 out of 15 in a big experimental sample, rejection or acceptance of the hypothesis must be quite as carefully considered as if an equal probability estimate had been obtained from a small sample. Vice versa, when calculations show that in a small sample the probability of obtaining the observed result by chance is about 1 out of 10,000, the significance is quite as good as if it had been obtained from a sample 100 times as large. Another thing is of course that only perfectly randomized conditions justify the above statistical considerations. If bias is involved, which admittedly may in some kinds of experiments happen more easily with a small sample, no statistics can remedy the false results.

Experimental Treatment.

When the effect of some drug is studied, it is frequently done by giving injections to the experimental animals, leaving the controls untreated. However, it may be that the effect thus obtained is not specifically due to the particular substance injected, but rather to the injection. Therefore, it is correct to inject also the controls at the same time with the solvent in which the drug is dissolved.

The same principle applies to all other kinds of experiments. Serious errors may follow if the treatment of the experimental animals and the controls is not in all respects identical except for the variant under investigation. This may sometimes be rather difficult. Suppose that the effect of low atmospheric pressure is under investigation. The experimental animals are put into a low-pressure chamber while the controls are left outside. In this case, painstaking trials must be done to make sure that the environment is similar in other respects than the pressure. The illumination conditions may be radically different in the low-pressure chamber and in the room where the control animals are kept. Likewise, the influence of factors such as noise, temperature, humidity, etc., may cause changes in the animals not due to low pressure.

Killing of Animals.

The schedule of randomization must rigidly be followed also during the killing. Killing first the control group and then the experimental animals, or vice versa, may artificially induce changes superimposed on the experimental changes. Such an effect is pronounced, if the killing is done at long intervals, as the environmental conditions may change.

Production of systematic error of this kind can be prevented by killing controls and test animals alternatively. However, although animals in both groups are then equally influenced, the experiment may still be ruined. It has, e.g., been demonstrated that even very minor stimuli may be followed by a drop in the ascorbic acid content of the adrenals. If the animals to be killed are transferred to another room, the ascorbic acid content will decrease progressively. Thus, the animals killed first show a higher ascorbic-acid content than the last killed animals. Although experimental animals and controls are now equally affected, this difference may completely mask any minor alterations due to the factors under study.

This applies also to the method of killing. In many laboratories it is customary to kill the animals by a blow on the head and bleed the animals through the neck. It can be shown that such method of killing may induce differences in the blood content of various organs. Depending on the strength of the blow, the circulation of the animal is variably affected, and the bleeding after cutting the neck is accordingly more or less complete. In most instances it is quite convenient, and least painful to the animals too, to cut the neck without any previous treatment. With some experience it is not difficult to have the organ to be examined ready frozen one minute or so after the animal has been taken from the cage. Straightforward decapitation is frequently superior to methods involving any treatment, e.g. anaesthesia, before the actual killing.

In small laboratory animals such as the rat and the mouse decapitation can be done with sharp scissors. In larger animals it may be worth while devising a guillotine by means of which the animal can be suddenly killed without any previous treatment. *Glick, Swigart, Bahn* and *Hastings* (1954) recommend shooting of the animals in their own cage.

In certain instances, e.g. when the blood content of some organ is to be examined, anaesthesia before the killing may nevertheless be preferable. It is probably impossible to avoid small alterations in the distribution of blood in various organs, but if the procedure of anaesthesia is carefully standardized, comparable results may be obtained. *Davis, Plotz* and *Plotz* (1953) have published an elegant method for the study of the blood content of the adrenals. They exposed the adrenals during a light anaesthesia. The glands were frozen *in situ* by means of a spray of isopentane cooled with liquid nitrogen. The organs were transferred frozen into a freeze-drying equipment, where they were dried. The dried organs were then embedded in celloidin. This method is doubtlessly most adequate for the study of the distribution of blood. However, radical experimental changes in the blood distribution can sometimes be adequately demonstrated even if the animals are killed by cutting the neck. This has clearly been demonstrated in the kidney by *Trueta, Barclay, Daniel, Franklin* and *Prichard* (1947). Moreover, the vascularization can be investigated by injecting Indian ink into the blood vessels.

Taking a Tissue Sample.

For most purposes the above-mentioned freezing method is free of any objections. It is, however, rather elaborate and needs special apparatus and plenty of time. Simple dissection is therefore usually preferable. If the organ is small, it is usually best to cut it out as a whole. Care must be taken not to handle the organ excessively, because this may cause displacement of the substances to be examined or even alter the cytological picture. Dissection must not be unduly prolonged because post-mortem changes may quickly affect the result. If the exact form of a small organ has to be preserved, it may be advantageous to remove it with the surrounding organs and fix it with them. Otherwise, dissecting the organ free of adjacent adipose tissue is preferable.

If the organ or tissue to be examined is large, the problem of sampling is again entering. Consider e.g. a study of the nuclear size in the liver. Even in the mouse the liver is too big and a representative sample must be removed. Even if it has been demonstrated that nuclei are similar in all parts of the liver it is wise to take a piece from the same lobe and moreover from the same part of the same lobe in each animal, because different lobes may be differently affected in the experiment.

Due attention must also be paid to the direction in which the tissue piece is cut out. In the prostate gland, e.g., the histological properties are quite different at different distances from the urethra. To be able to make comparable specimens it is therefore necessary to cut the sections perpendicularly to the urethra. To be able to do this needles introduced into the urethra can be used. The orientation is frequently made easier by taking a piece of the adjacent tissues. Thus the prostate gland can be prepared out with all the accessory urogenital organs. The problem of orientation is then taken up first after fixation, when handling of the tissues is easier.

CHAPTER III

Preparation of Microscopic Specimens

Introduction.

When the tissue piece has been removed, it has to be worked to a specimen which can be examined under the microscope. To this end different procedures can be used. The most commonly used way is to cut sections from the organ. Cutting of sufficiently thin sections is possible only if the organ is made solid enough. In ordinary histology this is done by fixation and embedding in a solid medium. In histochemistry the same method can be used, but it is frequently preferable to cut the tissue fresh. Besides the cutting methods many other methods have been developed. One of the most useful ones is differential centrifugation. All these methods have been treated in many excellent books of histological and histochemical techniques and the reader is referred to these sources (*Lillie*, 1948; *Romeis*, 1948; *Glick*, 1949; *Lee*, 1950; *McClung Jones*, 1950; *Cowdry*, 1952; *Gomori*, 1952; *Lison*, 1953; *Pearse*, 1953). Some points which are important from the quantitative point of view will nevertheless be dealt with.

The Linderstrøm-Lang Technique.

Sections can be cut from a fresh tissue which is frozen hard enough. The knife must also be cool. *Linderstrøm-Lang* (1952) has developed a technique in which a rocking microtome installed inside a cold-box is used. The box is kept at a low temperature with the aid of a thermostatic device. This consists of a fan blowing air on solid carbon dioxide. When the fan is working, the temperature in the box decreases. The fan is controlled by a contact thermometer. As the temperature of the whole system is constant, sections of the frozen tissue can be cut without interruption. Constant cutting speed, which has been shown to result in uniform thickness of the sections, is easily maintained with the rocking microtome. Every other of the sections is used for histochemical or histological procedure, every other for chemical estimation. As the temperature in the box is kept low, the humidity also keeps low. The sections may therefore be dried in the box. Manipulations inside the box are done with hands in cold-isolating gloves. A heated glass window allows visual control.

The Linderstrøm-Lang technique requires special equipment and considerable amounts of solid carbon dioxide. The present writer has used a simpler technique in principle similar to that of Linderstrøm-Lang. Cutting is nevertheless done on an ordinary freezing microtome with a cooled knife. Individual sections are transferred frozen into small holes in a disk-shaped section holder about 1 cm. thick. The disk is kept at —78° C in a beaker surrounded by another beaker filled with solid carbon dioxide. The disk with the sections is transferred after cutting into a simple freeze-drying device shown in Fig. 4 (*Eränkö*, 1954). Sections of 30 μ are dry in about 30 min. Fresh-frozen sections may also be fixed in some chemical

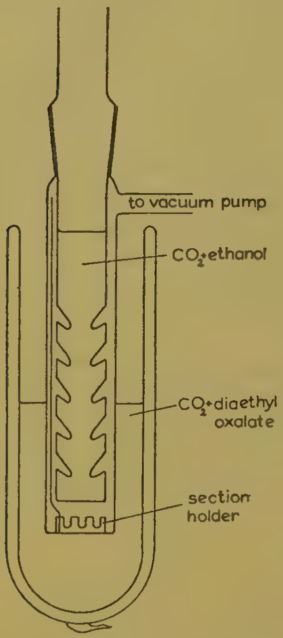


Fig. 4. Freeze-drying apparatus (*Eränkö*, 1954). The inner tube, cooled to —78° C with dry ice and alcohol, serves as a cold trap collecting water molecules escaping from the sections, kept at ca. —40° C with a mixture of dry ice and diaethyl oxalate.

fixative such as 10% formalin (4% formaldehyde) or they may be immediately transferred on the slide where they are allowed to thaw and dry. In some histochemical techniques it is necessary to float fresh-frozen sections directly on the reagent.

Freeze-Drying of Tissue Pieces.

Although cutting of fresh-frozen sections with an ordinary microtome is not very inconvenient, this method is not handy if a large number of pieces is to be cut. In such instances it is preferable to fix the tissues in pieces by some other means such als freeze-drying or chemical fixation. These methods have the additional advantage that thin sections, which are difficult to cut from fresh material, can be easily prepared.

Freezing of the tissue must be sudden as gradual freezing is accompanied with formation of ice-crystals. These ice-crystals cause deformation of cytological details and dislocation of substances in the organs. Earlier, liquid air was the most popular fluid for quenching the tissues. Owing to the poor heat-conductivity of vaporized air surrounding the tissue piece in liquid air, isopentane cooled to liquid air temperatures has recently been used by most investigators. Cooling of any inflammable fluid with liquid air is hazardous. Whenever possible, liquid air should therefore be replaced by liquid nitrogen. When the tissue is quenched between two metal surfaces, it is possible to use solely liquid air or solid carbon dioxide. Two metal pieces, conveniently attached to the end of long forceps, are first cooled in either liquid air or with the aid of solid CO_2. Meanwhile a thin slice of fresh tissue is cut. The slice is quickly transferred on one metal disk and the forceps are closed and put into liquid nitrogen or any fluid cooled with solid CO_2. Good heat conductivity of metals guarantees a rapid quenching.

The frozen pieces can be left for a while in the quenching fluid or in solid CO_2. When all pieces have been quenched, they are transferred into a freeze-drying equipment. Several designs of freeze-drying equipment have recently been published. With the best apparatus tissues can be dried in a few hours at tissue temperature as low as —60° C. In such equipment the tissue is dehydrated in a vacuum produced with a combination of mechanical and oil diffusion pumps. In the simple apparatus illustrated in Fig. 4 complete drying of tissue pieces not exceeding 1 mm. in thickness can be done in ca. 24 hours. The length of the drying period must be adjusted for each particular tissue. Tissues containing large amounts of lipids such as the brain and the adrenal require longer drying periods than other tissues. The lower the temperature of the tissue pieces, the longer is the drying process. For most purposes it is adequate to keep the tissue at about —40° C.

When the drying is completed the tissues can be either directly embedded in paraffin in vacuum or immersed in some fixing solvent such as alcohol. The temperature of the tissue is first allowed to rise to room temperature. Tissues to be embedded directly in paraffin under vacuum must be completely dry. Embedding in paraffin may indeed be used as a test of the dryness of the tissues. When paraffin is molten and the piece of tissue is falling in it, no air bubbles appear if the tissue is completely dehydrated.

Freeze-dried tissues may also be embedded in carbowax, which contrary to paraffin does not dissolve lipids. Some freeze-dried tissues can be cut without any embedding at all. Further information can be obtained from several recent articles dealing with freeze-drying (*Scott* and *Hoerr*, 1950; *Stowell*, 1951; *Bell*, 1952; *Glick* and *Malmström*, 1952; *Pearse*, 1953; *Eränkö*, 1954; *Moberger*, *Lindström* and *Andersson*, 1954).

Chemical Fixation.

A large number of chemical fixatives is in current use. Many of them can, nevertheless, be substituted with 10% formalin, which appears to be the most useful fixative of all. Formalin preserves well not only the cytological details but also many lipids, proteins, enzymes, and even many polysaccharides. Formalin can also be frequently used although some special fixative is ordinarily prescribed. Mordanting of formalin-fixed sections in the specified fixative remedies the situation. Frozen sections can be easily prepared from formalin-fixed tissues. For other fixatives consultation of the various volumes dealing with histological techniques is recommended (see page 33).

If the tissue has been fixed in an aqueous fixative, it has to be dehydrated before infiltration in paraffin wax. Dehydration can be done with the aid of a series with increasing concentrations of ethyl alcohol, later mixed and replaced with butyl alcohol, which can be mixed with paraffin. The following schedule is given as an example: 10% formalin overnight, 50% ethyl alcohol 12 hrs., 70% alcohol 12 hrs., 96% alcohol 12 hrs., 96% ethyl alcohol and butyl alcohol equal parts 6 hrs., two changes of butyl alcohol 6 hrs., two changes of paraffin wax 2 hrs., embedding in paraffin wax. If the tissue pieces are kept in continuous movement in the fluids, the procedure can be greatly abridged. Further, the minimum length of treatment in the fluids essentially depends on the size of the pieces.

Differential Centrifugation.

As mentioned earlier, other methods than section cutting may be used for making preparations thin enough for microscopical studies. By means of differential centrifugation it is possible to separate different tissue and cell constituents thus making their chemical analysis possible (*Bensley* and *Hoerr*, 1934; *Claude*, 1944; *Dounce*, 1950; *Schneider* and *Hogeboom*, 1951). This method has been recently much used for the chemical analysis of cytoplasmic particles. Full treatment and description of this method falls outside the scope of this book, but it may be mentioned that in our experience differential centrifugation in hypertonic sucrose solutions at 0^0 C gives a good separation of nuclei and cytoplasmic granules. Apparently, the chemical composition of these constituents is not as much altered as would be expected. See also *Glick* (1949) and *Cowdry* (1952).

CHAPTER IV

Specimen Thickness

Introduction.

To be able to express the results of quantitative measurements in terms of unit volume of tissue, the thickness of the microscopical specimen must be known. When sections are used, an estimate of the section thickness is given by the adjustment of the microtome. Unfortunately, the section thickness is frequently subject to considerable variations although everything is done to keep the thickness constant. Unless these variations are taken into consideration, otherwise adequate measurements may be invalidated.

If the quantitative measurements are carried out on relatively large areas of the section, the error introduced by variations in the section thickness can frequently be reduced enough by measuring the average thickness of the section simply by weighing it. If, on the other hand, the measurements are carried out on small tissue constituents, uneven thickness in different parts of the section may be a source of error which cannot be remedied by measuring the average thickness. In such instances it is preferable to measure the thickness of the specimen in restricted areas. This can be done mechanically in sections hard enough. The best method of estimating thicknesses in small areas is nevertheless based on optical measurements.

It must be realized that errors due to the lack of uniformity in section thickness cannot always be eliminated by thickness measurements. Thus, the intensity of many histochemical reactions is not linearly proportional to the section thickness (see pages 45 and 114), wherefore it may be necessary to reduce thickness variations to the minimum. In such instances, thickness measurements are of great value in the selection of equally thick sections.

Weighing of Sections.

Any balance sensitive enough is adequate for weighing the sections. Such a balance can be fairly easily constructed. Fig. 5

shows a string balance modified from that published by *Trevan* (1926). It makes use of the fact that bending of a steel wire is within a reasonably large range proportional to the bending weight. The displacement is in this balance determined with the aid of a micrometer screw. When the steel thread touches the micrometer screw, an electric circuit is closed and the indicator lamp lights up. Depending on the cross section and on the modulus of elasticity of the material used, the sensitivity range of this kind of balance can be suitably adjusted. The reading can also be done optically with a kathetometer or a horizontal microscope with an ocular micro-

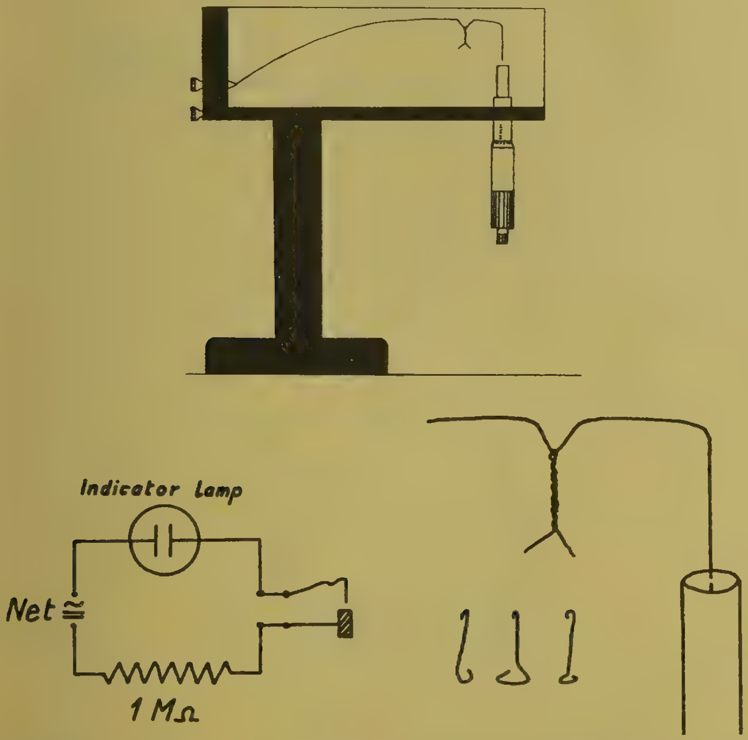


Fig. 5. A steel-wire balance modified from that designed by *Trevan* (1926). The displacement of the wire caused by the object is read with the aid of a micrometer screw. The deflection is directly proportional to the weight within a wide range.

meter. If the steel wire is replaced with a hollow quartz fibre (Fig. 6), highly sensitive balances can be constructed (*Lowry*, 1941, 1953). Instead of quartz, fine glass capillaries can also be used. The wire or fibre used for weighing must be enclosed in a box to prevent the influence of air currents. To avoid the effect of static potentials the box has to be made of some conductive material and it must be earthed. Some radioactive paint in the inner wall of the box facilitates ionization.

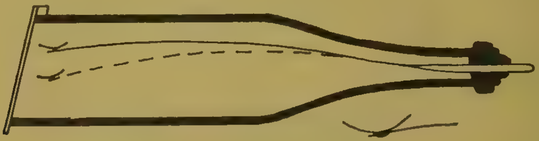


Fig. 6. Fishpole quartz balance, sensitive down to 0.01 μg. (*Lowry*, 1953). The inside of the glass case and the fibre are platinum-coated. The quartz rod is fixed into the case with Wood's metal. The pan is made of glass, thin enough to show interference colours, by blowing out a glass bubble rapidly. It is sealed in place with a minute amount of hard de Khotinsky cement. In the present writer's experience Pyrex glass can be used instead of quartz for making the fibre. Displacement is read through the window with a horizontal microscope fitted with an ocular micrometer.

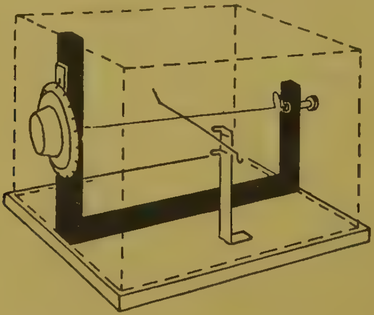


Fig. 7. A simple torsion balance (*Strong*, 1949). The torsion fibre and the cross arm are made of quartz. To maintain the tension of the torsion fibre constant a quartz bow is used. The weight of the object is read from the dial after twisting until the cross arm gives a zero reading on the divided head. The sensitivity depends on the thickness of the quartz fibre. With thin fibres a sensitivity of 0.1 to 0.001 μg. per scale division can be reached.

Another type of sensitive balances makes use of torsion principle. Fig. 7 shows a torsion balance, which may be constructed of quartz fibre (*Strong*, 1949). It is not difficult to construct an accurate balance sensitive to less than 10^{-6} gm. See also *Lowry* (1949).

Mechanical Thickness Measurements.

By weighing tissue sections it is only possible to know the average thickness. According to *Glimstedt* and *Håkansson* (1951), however, marked variations may occur in the thickness of different parts of the same section. These authors modified a mechanical meter, originally constructed for tolerance controls in the engineering industry, for the measurement of section thickness. Their instrument works with a minimum pressure of 200 mg. In measurements of paraffin sections, using a sphere of 6 mm. diameter in the meter, no noticeable deformation of the section was observed (*Glimstedt*, personal communication). It should be noted, however, that a pressure of 200 mg. is too large to allow measurements on small areas; 200 mg.'s weight on an area of 100 × 100 μ corresponds to a pressure of 2 kg./cm^2.

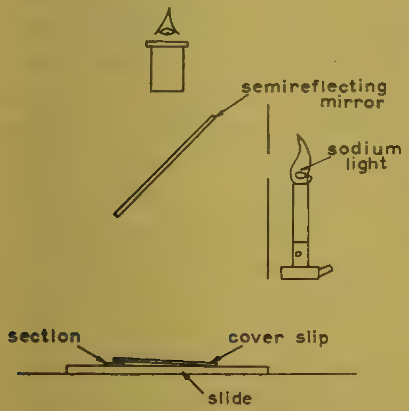


Fig. 8. Schematic picture illustrating the interference method for measuring the section thickness (*Richards*, 1947). Both the cover slip and the slide must be optically flat. Examined and illuminated with monochromatic light from above, definite interference fringes are seen. The section thickness is obtained by multiplying the number of these fringes by 0.29, when sodium light is used.

Optical Thickness Measurements.

Several optical methods for the estimation of section thickness have been described. One of them is to focus on the lower surface of the cover slip and subsequently on the upper surface of the slide, the difference being read from the scale of the fine movement screw of the microscope. The accuracy of this method is low. *Caspersson* (1950) has described a method based on refractive index differences. The original reference should be consulted. Fig. 8 illustrates an interferometric method (*Richards*, 1947).

Determination of Dry Mass.

Recently, two useful methods have been described. They are *X-ray absorption measurement* (*Engström*, 1946) and *interference microscopy* (*Dyson*, 1950). If very soft X-rays are used, the absorption is dependent on the dry mass of the tissue (*Engström* and *Lindström*, 1949, 1950). An X-ray absorption image can be prepared with a resolving power of about 1 μ. This method unfortunately suffers from complicated design of the apparatus required.

Equally valuable information can be obtained with the aid of the interference microscopy in a fairly simple way. The method makes use of cell refractivity or optical path-length, which is a measure of the total mass of substances other than water, that is the dry mass. Results obtained with X-ray absorption and interference microscopy have been recently compared in a variety of biological structures (*Davies, Engström* and *Lindström*, 1953). A good agreement was observed between the two methods. The interference method has the particular advantage that it may also be used for studying quantitatively changes in the dry weight of individual cells, which is impossible with any other method. Further, the resolving power of the interference microscopy is much superior to that of the X-ray absorption technique. Alternative dry-mass determination and light absorption measurement can be done with relative ease. Interference microscopy is discussed in further detail on page 48.

Linderstrøm-Lang and other members of the Carlsberg group have devised techniques for the estimation of so-called "reduced weight" in small tissue samples. Reduced weight is the weight of the sample minus the weight of an equal volume of water. For details see *Linderstrøm-Lang* (1952).

CHAPTER V

Microscopic Demonstration of Tissue Details

Introduction.

In most instances, untreated tissue sections or smears show little or no details when examined under an ordinary microscope. Special methods are therefore necessary to demonstrate the details. This can be done by treating the section with histological staining methods or with histochemical techniques. Even unstained specimens can be examined, if use is made of the absorbing properties, of the fluorescence, or of the refracting index differences in various tissue constituents. Tissues labelled with radioactive indicators can be used for the same purpose.

Histological Staining Methods.

Histological staining methods are either progressive or regressive, or mixtures of these. In progressive methods the specimen is immersed in a staining solution until the desired coloration is obtained. In regressive methods the tissue is first overstained and excessive stain is removed in a liquid dissolving the dye. In most regressive staining methods it is customary to control the decolorization of each individual section visually. The differentiation is finished when a satisfactory stain is obtained. Mixtures of these principal methods are common. Thus, in staining methods involving the use of several dyes, the first step may be regressive while other stains are thereafter applied in a progressive manner.

In quantitative studies a staining may be necessary

a) to make details visible (e.g. in nuclear measurements),

b) to stain differentially some tissue constituents (e.g. for the estimation of the numbers of basophilic and eosinophilic cells in the pituitary gland),

c) to be able to study quantitatively the stainability of some tissue constituent (e.g. the intensity of basophilia in the cytoplasm of nerve cells).

The conditions which a staining method must fulfil are accordingly different in each case. When morphologically defined characteristics are investigated, variations in the intensity or colour of different tissue elements may be of little importance, and any general staining method is adequate. When measurements on differentially stained tissue elements are in question, care is necessary to avoid errors due to variations in the staining technique. To mention one example, Mallory-Heidenhain's azocarmin stain is commonly used for differential staining of the cells of the anterior lobe of the pituitary. In this technique, the tissue is first overstained with azocarmin, differentiated under visual control, overstained in a mixture of aniline blue and orange G and differentiated again. The final result thus essentially depends on two subjectively controlled stages, and it is easy to show that similar cells, e.g. erythrocytes, can be stained either red or blue with the same technique when the subjectively controlled stages are slightly altered. It is clear that with such techniques any experimental changes may be completely masked or, on the contrary, differences may be falsely created in specimens actually identical. Therefore, all steps must be so standardized that no space is left to subjective judgment.

When the intensity of the staining is to be measured, still more rigorous control of all steps in the staining procedure is necessary. The final result is sensitive to small variations in the fixation and embedding procedures, in the dye concentration, in the acidity, ionic strength and age of the staining solution, in the lengths of staining, washing and dehydration periods, etc. Reproducibility of the technique must therefore be carefully checked before it is used for quantitative purposes. For comparison of sections from controls and experimental animals, pairs of sections from both groups should be treated attached on the same slide. Progressive staining methods are mostly preferable.

Theoretical aspects of histological staining have been discussed in *Zeiger*'s (1938) excellent monograph. An admirable review of factors controlling the staining of tissue sections with acid and basic dyes has recently been published by *Singer* (1952). An abundance of staining techniques has been described in books by *Lillie* (1948), *Romeis* (1948), *Lee* (1950), *McClung Jones* (1950) and *Cowdry* (1952).

Histochemical Reactions.

The aim of histochemical techniques is to demonstrate in tissue the sites possessing some specific chemical property or properties. After the complete procedure, these sites are visible either dark or coloured. An ideal histochemical method would be specific to the substance it tries to demonstrate, and the distribution and intensity of the visible reaction product would truly indicate the location and concentration of this substance in the living cell. Unfortunately, there is not a single histochemical test completely fulfilling these conditions. The specificity of most histochemical techniques is limited, a group of substances being responsible for the staining reaction. Considerable losses of the substance to be demonstrated may occur during the treatment of the specimen before and during the histochemical procedure. Moreover, not only falsely negative but also falsely positive results may be caused e.g. by displacement of substances during the treatment. Furthermore, the intensity of the reaction produced is rarely in a simple relation to the concentration of the reactive material present in the section. "All-or-none" reactions are frequent, i.e. subthreshold concentration of the substance to be demonstrated does not produce any reaction at all while a slightly higher concentration above the critical threshold produces a maximum blackening. Even if the responsible substance has survived the procedure and remained in its original site in the tissue, misleading distribution of the visible reaction may be caused by dislocation of the reaction product to neighbouring parts of the tissue having an affinity to the product.

Bearing the above facts in mind, it is clear that before using any histochemical method the specificity of the reaction should be tested, and due attention should be paid to the possibility of decomposition or diffusion of the substance to be demonstrated, of any intermediate reaction product, and of the final visible compound. Unless this is done, the slides, though nicely stained, may be entirely valueless from the histochemical point of view. A surprisingly large proportion of published histochemical methods is without much value and only a few of them have been fully critically studied. Fortunately, three excellent books of histochemistry have recently appeared (*Gomori*, 1952; *Lison*, 1953; *Pearse*, 1953) in which a large variety of methods is examined from both theoretical and practical points of view.

If a histochemical method is reasonably specific and if there are reasons to suppose that diffusion processes do not invalidate the technique, photometric measurement of the resulting darkening or colour never does any harm. Much valuable information can be obtained in this way, though the relation between the intensity of coloration and the concentration of the substance responsible were unknown. However, if the staining intensity is to be expressed in terms of concentration or of chemical activity, relation between these two variables must be carefully examined. Sometimes it is possible to check directly the results by ordinary chemical methods. In most cases, however, this is not the case. Coujard slides (see page 113) may be useful in such instances.

The necessity of maintaining standard conditions of treatment, above emphasized in connection with the histological technique, applies at least equally well to the histochemical techniques. Also in this case it is of advantage to treat experimental and control specimens on the same slide.

Absorption Methods.

Histological staining techniques and histochemical methods are used to produce in sections sites absorbing visible light. Although a section is completely colourless when examined in transmitted visible light, some of its constituents may selectively absorb rays outside the visible range. Such details can be brought out by examining them in ultraviolet light (see *Barer*, 1950 a, b; *Caspersson*, 1950; *Barer*, *Holiday*, and *Jope*, 1950), in infrared light (*Barer*, 1950 a, 1951, 1952 d), with X-rays (*Engström*, 1946; *Engström* and *Lindström*, 1949, 1950), or by means of the electron microscope (*Scott* and *Packer*, 1939 a, b; *Scott*, 1943). Many biological substances have characteristic absorption peaks in the ultraviolet and their distribution in the specimen can be studied by ultraviolet photomicrography. X-rays and electrons can also be used in the same way, and they have been found particularly useful for the demonstration of the distribution of various minerals. Little work has hitherto been done with microspectrography in the infrared region.

All these methods require expensive and complicated equipment. Absorption microspectrography can in a simpler way be used for the study of stained sections in ordinary light. This technique is described later in this volume (chapters VIII, IX, and X).

Fluorescence Microscopy.

Many biological substances are autofluorescent, i.e. when illuminated with ultraviolet light they emit light. Based on this fluorescence, distribution of some substances can directly be observed in fresh tissues. Though the substances responsible for the fluorescence would not be known, fluorescence microscopy may sometimes be useful for demonstration of different kinds of tissue in an unstained preparation.

The intensity and the colour of the fluorescing light are dependent on the nature and concentration of the fluorescing substances. Fluorometry is indeed a convenient method for quantitative determination of some substances. Unfortunately, this applies only to pure solutions. Even very minor alterations in the composition of the solvent in which the fluorescing substance is dissolved may strongly modify both the wavelength and the intensity of the fluorescence (*Pringsheim*, 1949). Therefore quantitative estimation of fluorescing substances in microscopical specimens is connected with many difficulties. It has e.g. not been possible to identify the spectrum of pure vitamin A in tissue sections, although an abundance of presumptive evidence has been collected indicating that vitamin A is responsible for a strong fluorescence in many tissues (*Popper*, 1944). *Sjöstrand* (1946 a, b) nevertheless succeeded in identifying by fluorescence microspectrography the cytological localization of riboflavin and thiamin.

Any ordinary microscope can be used for fluorescence studies, provided that its condenser and other lenses do not show excessive fluorescence. Carbon-arc or mercury-vapour lamps with high surface-light intensity are adequate for the purpose. A cuvette with 5% copper sulfate and a Wood-glass filter are used for obtaining ultraviolet light. Any yellow filter causing a complete extinction of this light can be used as a stop filter.

Contrast and Interference Microscopy.

Although most tissues do not absorb visible light, i.e., they leave the amplitude of the light radiation unchanged, differences in the refractive indices in various parts of the tissues cause changes in the phase of the light rays. The magnitude of these phase changes is dependent on the refractive index and on the thickness of the tissue through which the light has to pass. In interference and phase-contrast microscopes, light rays in which a phase shift has been caused by refractive tissue particles are allowed to interfere with light rays in which no such shift has occurred. As a result, lighter and darker areas corresponding to the refractive index differences are seen in the tissue. While the phase shift is in the phase-contrast microscope merely used as a means of making the tissue visible, the magnitude of the phase shift can be quantitatively measured in the interference microscope. (For literature see *Barer*, 1949, 1952 a-d; *Richards*, 1949, 1951; *Dyson*, 1950.)

A new kind of contrast microscope has recently been constructed by *Wilska* (1953 a, b, 1954; see also *Barer*, 1953 a). This "anoptral-contrast" microscope has been found useful particularly in the study of very small details, which are seen better than in the phase-contrast microscope.

These microscopes offer interesting possibilites for quantitative refractometry (*Barer*, *Ross*, and *Tkaczyk*, 1953). In all of them, particles with a refractive index different from that of the surrounding medium appear either brighter or darker than this medium. If albumine solution is used as a mounting medium, the refractive index of this can be varied without altering the osmotic pressure, and the refractive index of any tissue particle can be determined by matching with that of the albumine solution. When the refractive index difference is zero, the particle virtually disappears. In a negative phase-contrast microscope a particle whose refractive index is higher than that of the medium appears dark, and vice versa. The mounting medium to be used for refractometry should not of course penetrate the particle. For the study of living cells it must further be non-toxic and not impair their structure or function.

It is fortunate that the specific refractive increment, i.e. the increase of the refractive index per 1% increase in concentration, is practically equal for all protein solutions. Even mineral and carbohydrate materials have specific refractive increment values of the same magnitude order as proteins. By measuring the refractive index of some tissue constituent it is therefore possible to get a fairly accurate idea about the concentration of dry substances in this constituent. The particular advantage of this measuring method is that it is suitable for measurements in living cells. Phase-contrast, anoptral-contrast, and interference microscopes can equally well be used.

If the last one is available, the total phase shift can be measured. As this shift is linearly proportional to the amount of refractile substance and inversely proportional to the specific refractive increment, interference microscopy is, as mentioned earlier, a convenient method for estimating the total dry mass in different parts of the section (*Barer*, 1952 c; *Davies* and *Wilkins*, 1952; *Davies*, *Engström*, and *Lindström*, 1953). With the aid of this method quantitative microspectrographic analysis can be related to the dry mass of the corresponding parts of the tissues or cells. Moreover, by combining this method with thickness measurement it is possible to estimate refractive indices and concentrations in tissue elements without the use of various immersion media.

Radioautography.

Sections cut from organs taken from animals injected with radioactive substances are capable of creating images when placed on a photographic plate. The area darkened in the developed plate shows the localization of the radioactive substances, and the darkening is dependent on the intensity of radioactivity in each site. If a maximum resolution is sought, the sections can be impregnated with the sensitive emulsion. The theory and methods of the radioautographic localization of radioelements in tissues have been admirably discussed by *Gross*, *Bogoroch*, *Nadler* and *Leblond* (1951).

Köhler's Illumination.

Proper use of the microscope is one of the main conditions for the success of quantitative microscopic studies. *Barer*'s (1953 b) guide is a valuable introduction to everybody who wants to learn the adequate way of using the microscope. Correct illumination must be used, even if the quantitative studies are not primarily concerned with changes in the light intensity.

For practically all purposes, including those in which quantitative light measurements are done, *Köhler*'s principle of illumination is adequate. This principle is illustrated in Fig. 9. Fig. 10 shows how

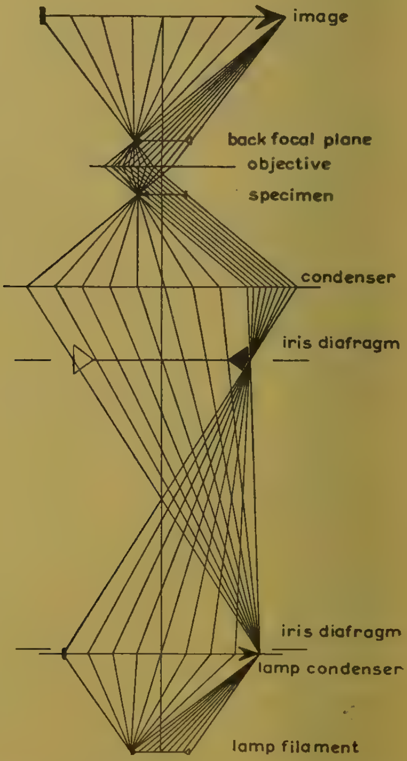


Fig. 9. Köhler's illumination. Note that images of the lamp filament are formed on the plane of the iris diaphragm of the microscope condenser and on the back focal plane of the microscope objective. Images of the lamp condenser diaphragm are on the specimen plane and the final image plane.

this illumination is reached in praxis. It may be observed that the image of the lamp filament is formed by the lamp condenser on the focal plane of the sub-stage condenser. Therefore, all light rays emerging from each point in the filament are going parallel through every point in the specimen. Vice versa, each individual point in the specimen is illuminated by every point in the filament. The diaphragm of the sub-stage condenser does not influence the magnitude of the illuminated area in the specimen but it controls the angle in which the light is reaching the specimen, working thus as an aperture diaphragm. By means of it, the numeric aperture of

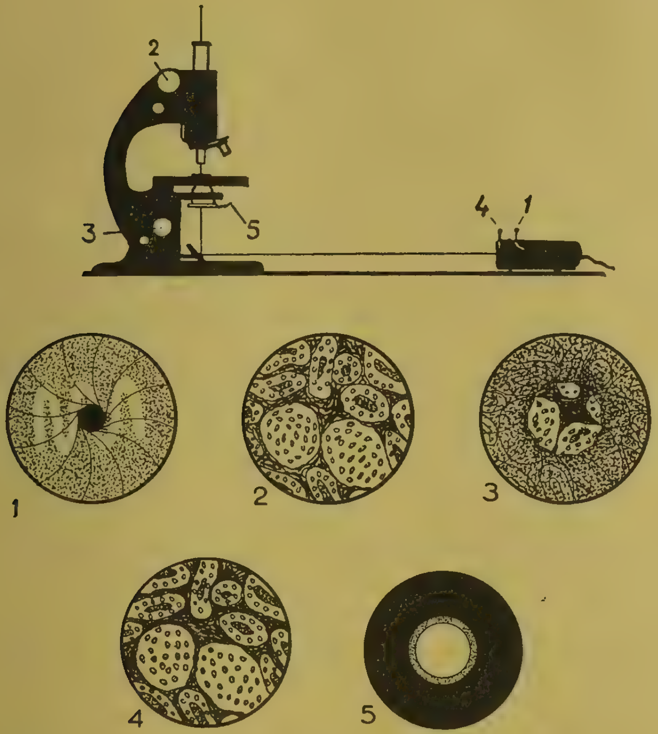


Fig. 10. Köhler's illumination in practice. 1) The image of the lamp filament is focused on the condenser diaphragm. 2) Looking in the microscope the image of the specimen is made sharp. 3) The image of the nearly closed lamp-condenser diaphragm is focused on the specimen plane by moving the microscope condenser. 4) The lamp-condenser diaphragm is opened until its border is just no more visible in the microscope. 5) With the ocular removed the back of the objective is examined and the diaphragm of the microscope condenser is closed until it is just visible.

the light bundle by which the specimen is illuminated is set somewhat smaller than that of the objective. As every point in the specimen is illuminated by every point of the filament it is not compulsory to have an even distribution of light in the filament plane.

The lamp diaphragm is sharply in focus simultaneously with the specimen. It works thus as a field diaphragm. The magnitude of the illuminated field in the specimen should not exceed that under examination, otherwise the disturbing effect of light scatter increases unnecessarily. As the resolving power of a microscope is dependent on the numeric aperture, care must be taken that, at high magnifications, a sufficiently large aperture of the condenser is used. If the image of the lamp filament on the sub-stage condenser diaphragm is smaller than the total diaphragm opening, this may not be the case; the situation can be remedied by increasing the distance of the lamp from the microscope. To use the full aperture of immersion objectives, immersion oil must also be used between the condenser and the slide. Without immersion oil the effective numeric aperture of the condenser keeps below 1.0, and the resolving power is diminished.

At low magnifications the microscope condenser usually illuminates a too small area in the section. Removal of its upper lens usually makes possible a correct illumination in a field large enough. When magnification is changed, the illumination must be adjusted every time. However, when low magnifications are used only for finding a suitable object for quantitative measurements at higher magnifications it may be unnecessary to adjust the illumination properly for the lower magnification.

In spite of its many advantages, Köhler's illumination has the drawback that only a relatively small fraction of the total light intensity of an ordinary microscope lamp can be used at higher magnifications. This situation can be corrected by decreasing the focal length of the lamp condenser. A lamp condenser in which the focal length can be altered by changing lenses is particularly useful when the magnification must be frequently changed. Such a lamp has been recently designed by *Barer* (personal communication) and it should soon be commercially available.

In Köhler's illumination the intensity of light must not be adjusted with the lamp diaphragm or the sub-stage diaphragm. If the intensity is excessive, neutral filters or adjustable lamp voltage must be used. At higher magnifications, particularly when relatively monochromatic light is used, the light intensity of the lamp is rarely too high. With Köhler's illumination it is mostly not necessary to insert any ground glasses in the system. If it is wanted to do so, the ground glass should be inserted between the bulb and the lamp condenser or immediately below the specimen, because then the loss of light is minimal.

Photomicrography.

Preparation of good photomicrographs is not more difficult than ordinary photography. When the illumination is properly adjusted and the image is sharply focused on the plane of the photosensitive emulsion, a correct exposure is about the only condition for a good photomicrograph.

As a good emulsion for general purposes, any panchromatic material with sufficient contrast is suitable. Panchromatic material has the advantage that with the aid of suitable filters it is possible to make a contrast-rich photomicrograph of microscopic details of any colour. For instance, when green tissue constituents are wanted to stand sharply out, a red filter is used. For ordinary photomicrography one yellow, one blue, and one red filter are sufficient.

The exposure time is dependent on the spectral distribution and the intensity of light entering the microscope, the microscope magnification, the distance of the photographic emulsion from the ocular, the absorbing properties of the microscopical specimen as well as the sensitivity of the emulsion. If no exposure meter is available, the correct exposure at each illumination condition and magnification must be found by trial and error. It is wise to standardize the illumination conditions and prepare a series of trial exposures at three different magnifications, each with three different kinds of filters, and taken of specimens having low, medium, and high absorption. When proper exposure times have been worked out for these 27 different conditions, preparation of first-class photomicrographs of any kind of specimen is bound to be successful even without an exposure meter. Any exposure meter with sufficient sensitivity is nevertheless a very useful aid in photomicrography. For the measurement the meter should be placed on the plane of the photographic emulsion.

The type and size of the photographic material in photomicrography is a matter of taste. Equally successful pictures can be taken on 35 mm. films as on large plates. In the first case the running costs keep low, while the use of plates is more expensive because of the higher price of the negative material. This drawback is frequently outbalanced by the facts that a simple and sufficient plate camera can be made of card-board at a low cost and that contact copies from plates are often sufficiently large.

A film or plate support for photomicrographic purposes can be made of an old camera. Any additional optics between the microscope ocular and the photographic emulsion is always superfluous and results in decreased sharpness. If the camera shutter is used, it is wise to have no rigid contact between the camera and the microscope, because vibrations caused by the shutter may result in blurring of the image. For most purposes it is advantageous to adjust the light intensity so that the exposure lasts ca. 5 sec.

In plate cameras, focusing is most conveniently done on a ground glass. The same principle may be applied to any type of camera, but for routine use a reflex camera is most convenient, see page 108.

The above instructions are merely valid for the preparation of photomicrographs in which quantitative measurements are performed on morphological basis. The photographic method of photometry has in further detail been described in chapter X, page 120.

Projection and Drawing.

In many instances the morphological measurements are most practically done on the projected image of the specimen. Fig. 11 shows a simple type of microprojector. For low magnifications an ordinary microscope lamp is perfectly adequate but for higher magnifications it may be necessary to increase the light intensity. Instead of the arrangement shown in Fig. 11 in which the microscope axis is horizontal, two mirrors or reflecting prisms may be used for projecting the area on paper, or the microscope may be fixed upside down above the paper. A further alternative is to project the image on a ground glass above the microscope, the image being examined from above. Such an arrangement has the advantage that the shadow of the examiner's hand or pencil does not disturb the drawing or measurement.

The projection method is somewhat impractical at higher magnifications. It is then more convenient to draw the pictures with some kind of drawing apparatus, one of which is exemplified in Fig. 12. In this instrument the microscopical image is seen simultaneously with the drawing paper beside the microscope. The inten-

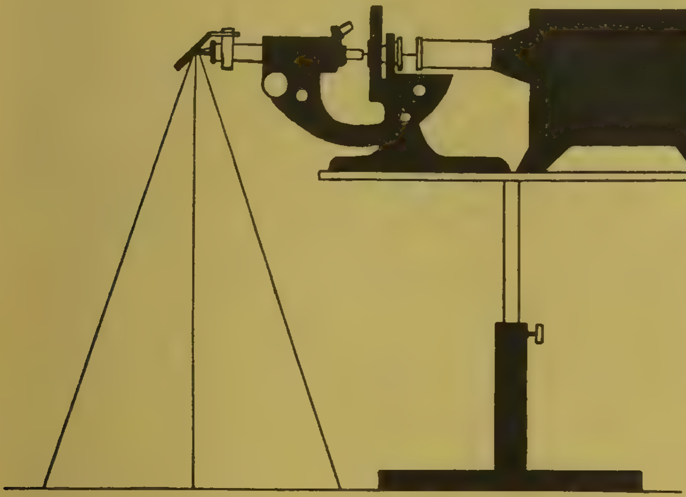


Fig. 11. A simple projection arrangement.

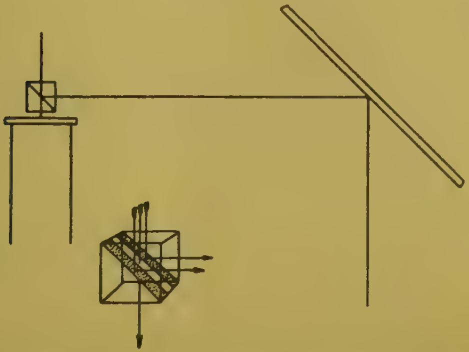


Fig. 12. A schematic drawing illustrating Abbe's camera lucida. With the aid of the half-reflecting prism the image of the specimen and the table nearby the microscope are seen superimposed, which allows accurate reproduction of the specimen.

sities of illumination in the microscope and on the paper must be matched to see both with a necessary accuracy.

Whatever method is used for transferring the microscopic image on paper, it is good to remember that frequently only the central area of the microscopic field is reasonably free of distortion. This can be demonstrated and its magnitude can actually be measured by comparing drawings or photomicrographs in the center and the periphery of the microscopic field.

Linear magnification is determined by replacing the specimen slide with a stage-micrometer without altering anything in the projection system. The distance between the lines in the magnified image of the micrometer scale is then measured. Knowing the true distance, frequently 100 or 10 μ, the linear magnification is easily calculated.

CHAPTER VI

Estimation of Relative Volume

Introduction.

Relative volume of some tissue is without doubt one of the most commonly measured variables in quantitative histology. Several methods are available for such measurements, each method being more suitable for some particular purpose than the others. All these methods give estimates of the relative *areas* in tissue sections, and the relative volumes are obtained by simple calculation. If only one representative section is measured in each animal, the estimate of the relative volume is equal to the obtained value of the relative area. If, on the other hand, several sections have been measured from the same piece and the proportional area in each individual section is p_i (i.e. area of the tissue to be estimated/total area of the section) and the total area of the section is a_i, a correct estimate of the proportional volume is then $p = \frac{\sum a_i p_i}{\sum a_i}$.

It must be noted that the section thickness may have an influence on the estimate of relative areas. This is the case if the tissue whose relative volume is to be assessed is very irregular and unstained in contrast to the surrounding tissues. In thick sections the chances to overlook considerable areas of the unstained tissue are then obvious. On the other hand, when the relative volume of, e.g., nuclei is estimated in thin sections, the relative number of such nuclear caps which are too thin to be seen increases and too low values are obtained.

Planimetric Measurements.

Area measurements can be very accurately done with a planimeter. The principle of planimetric measurements is illustrated in Figs. 13 and 14. To measure an area, the tracer point of the planimeter is placed on a selected starting point, reading of the measuring wheel is noted, the area is circumscribed in a clockwise direc-

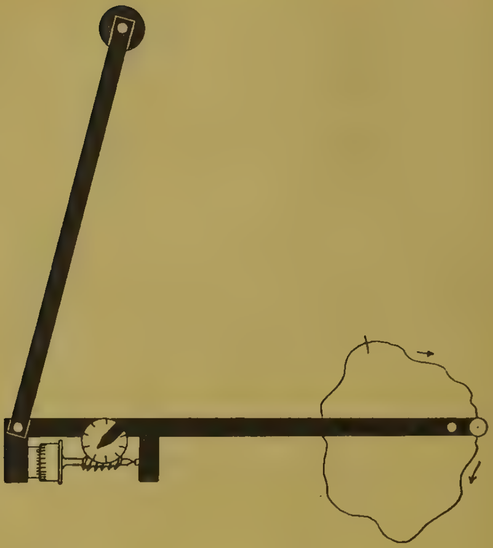


Fig. 13. The appearance of a planimeter.

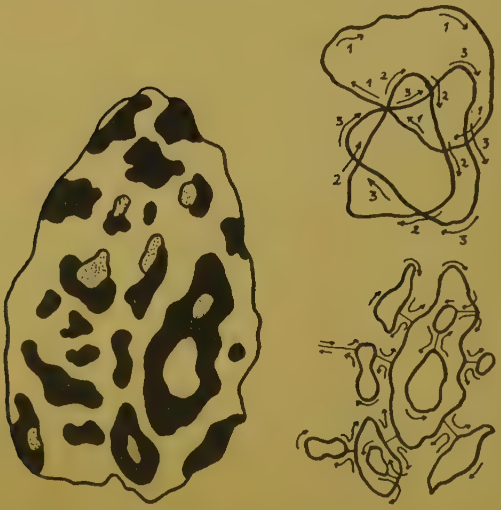


Fig. 14. The figure illustrates methods for summing the areas of small islets mechanically with the planimeter. The big advantage of the planimeter is that it is not necessary to measure separately the area of each individual "islet".

tion by means of the tracer point, and the reading of the measuring wheel is repeated.

Planimetric measurements can be done either on previously prepared drawings or photographs or directly on the magnified image of the section projected from below on a ground glass.

If a planimeter is not available, the area can be measured with comparable accuracy by drawing the outlines of the concerned areas on a paper. The corresponding pieces are then cut out and weighed. This method, which is very cumbersome, particularly when irregular structures are studied, has been much used in quantitative histology.

Planimetry is the most reliable method of area measurement and it is accordingly useful when high accuracy is necessary. Irregularity in the form and distribution of the specific tissue may indeed make planimetric measurements obligatory.

If a complete series of sections is prepared of a gland and every section is planimetrically measured, the obtained relative volume is not estimated but measured and the only sources of variability are the errors in measurements. The methods to be presented below give estimates of the relative areas in each section, the accuracy of which is essentially dependent on the *sampling* error. A sampling error is likewise introduced, if instead of a complete series of sections, representative sections only are measured planimetrically. Then the sampling variance within each section is zero but that between the sections must be considered. As the sampling variance is an important indicator of the accuracy of the applied technique, let us examine its nature in some detail.

Sampling Variance.

All proportion estimates can be expressed in the form

$$p = \frac{x}{y}. \tag{17}$$

in which x and y are random variables with no further specification. For example, when sections are planimetrically measured as above, x may be the area of some particular tissue and y the total area. As x and y vary from one section to another, p is also subject to variations. The variance of p can then be estimated from the following formula:

$$s^2(p) = p^2 \left\{ \frac{s^2(x)}{x^2} + \frac{s^2(y)}{y^2} - \frac{2r_{xy}s(x)s(y)}{xy} \right\}. \tag{18}$$

This formula is satisfactory provided that $s^2(x)$ is small as compared with x^2, and similarly for y. No special assumption is made of the variability of x and y. Therefore, the formula can be used in both systematic and random sampling if $s^2(x)$ and $s^2(y)$ are correctly estimated.

As the formula may seem complicated at first sight, let us analyze it in detail. In brackets, the first term includes the variance of x, $s^2(x)$. This divided by x^2 gives the square of the coefficient of variation of x. Similarly for the second term. In the third term, r_{xy} stands for the correlation coefficient of x and y. If x and y are independent of each other, r_{xy} will be zero, and the last term vanishes. The additional composition of this term is, except for the factor 2, the product of the coefficients of variation of x and y. Therefore, it is seen that the variability of p depends on the variability of both x and y and on their statistical interrelations.

Now, $s^2(p)$ divided by p^2 gives the square of the coefficient of variation of p, say, $V(p)$. Thus, denoting the coefficients of variation of x and y by $V(x)$ and $V(y)$, we obtain

$$V^2(p) = V^2(x) + V^2(y) - 2r_{xy}V(x)V(y). \tag{19}$$

In many applications the relative variabilities of x and y are equal, $V(x) = V(y) = V$. Then the formula reduces to

$$\begin{aligned} V^2(p) &= V^2 + V^2 - 2r_{xy}VV \\ &= 2V^2(1 - r_{xy}). \end{aligned} \tag{20}$$

Further it is seen that if there is a complete positive correlation between x and y, $r_{xy} = 1$, the variance reduces to zero. On the other hand, if the correlation approaches to zero, the relative variance amounts to $2V^2$. If $r_{xy} = -1$ (complete negative correlation), the relative variance equals to $4V^2$.

In many applications y is or can be kept constant, having thus no random variance. What remains in the formula (18) is then

(21) $$s^2(p) = \frac{p^2 s^2(x)}{x^2} = \frac{s^2(x)}{y^2}$$

i.e. the variance now depends on the variability of x alone. It should be noted that the larger y, the smaller the variance. In this case

(22) $$V^2(p) = V^2(x).$$

The reader should realize that the above formulas are of general importance and useful in many connections. No assumptions on x or y were made except that they are random variables, wherefore the formulas will in the subsequent discussion be used in alternative forms, giving x and y different meanings.

In the formula above sampling from an *infinite* population is assumed. If the population is finite, say N elements, of which n belong to the sample, the variance formulas should be multiplied by

(23) $$\frac{N-n}{N-1} = \text{finite multiplier.}$$

In a complete series of sections, each of which is planimetrically measured, $N = n$ and the sampling variance is equal to zero.

Area Sampling.

Frequently it is both elaborate and unnecessary to make a complete measurement in each section, e.g. when the relative volume of capillaries is to be estimated. In such case, measurements are performed in a number of visual fields or restricted areas of the visual field. If n visual fields or its fractions of constant area $\bar{y}$ are thus measured and the area of the specific surface in each visual field is x, the estimate of the relative area is

(24) $$p = \frac{\sum x}{n\bar{y}} = \frac{\bar{x}}{\bar{y}}$$

where $\bar{x}$ is the mean of x. In this case $\bar{y}$ is constant and consequently has no sampling variance, the variance of p being thus

(25) $$s^2(p) = \frac{s^2(x)}{n\bar{y}^2}$$

where $s^2(x)$ can be determined from the data according to equation (4), page 20.

The variance as given by (25) can be reduced by increasing the number of visual fields, n. Note, however, that the standard error is not proportional to n but to the square root of it. Therefore, if one wishes to cut the standard error in half, the number of fields must be quadrupled.

Formula (25) applies for *pure random sampling*, i.e. when new visual fields are thrown on the section randomly without regard to the earlier fields. The sampling can also be done by randomly selecting new fields so that they do not cover those thrown earlier. This represents a kind of random sampling without replacement, and it is clear that the more the area is sampled, the less there is unmeasured area left. If the whole section is covered, the estimate is no more an estimate but a measurement and, thus, has no sampling variance. Therefore, it is plausible to multiply formula (25) with a finite multiplier, in this case preferably $1 - \frac{a_0}{a}$ where a is the area of the section and a_0 the area already sampled.

Formula (25) involves a rule of a suitable magnification; the size of the field should be so determined that differences in x between grids remain small. This is generally the case if the field is large as compared with the configurations on the section. This rule in mind a visual inspection will give an idea of the optimal magnification. The rule represents a special case of a general rule which can be advantageously applied to many histological problems. This rule establishes that if the sampling units are totally measured (as here the grids) it is profitable to form the units so that they are internally heterogeneous and externally homogeneous, i.e. so that the variability between the units is rather small.

In systematic area sampling in which the section is covered with visual fields at equal distances, application of equation (25) will lead to overestimation of the sampling variance, if $s^2(x)$ is calculated over all the section. A plausible method for estimating $s^2(x)$ in this case is to use differences in x between neighbouring fields (*Yates*, 1949). Then the following formula is applied:

$$(26) \qquad s^2(x) = \frac{\sum d^2}{2n'},$$

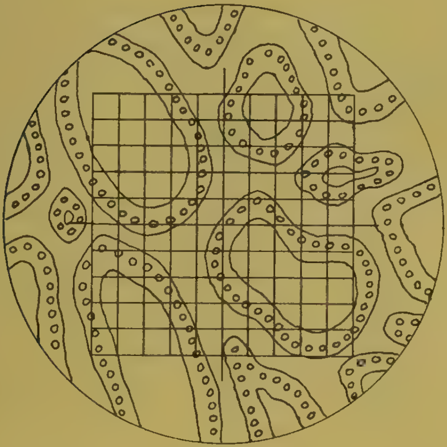


Fig. 15. A square-ruled network superimposed on a thyroid section for obtaining relative area estimates. Either the small squares are counted more than half-filled with the tissue whose relative volume is to be estimated, or the number of intersection points falling on this tissue is counted. Denoting epithelium with E, colloid with C, and stroma with S the following two results are obtained:

From points

C C C E S S C C E S E
C C C C S E C C E S E
C C C C E S E E S E S
E C C C E S S S E C C
S E C C E S E S S E S
S S E E S E C E S S S
E E S S S E C C E E E
E C E E S E C C C C C
E C C E S S E C C C C
E C C C E S S E E C E
E C C C E S E S S E E

C 44 or 36.4%
E 44 or 36.4%
S 33 or 27.2%

From small squares

C C C E S E C C S E
C C C E S E S E S S
C C C C S S E S E E
E C C E S S S S E E
E E E E S E E S S S
S S S S S E C E E E
E E E S E C C C C C
C C E S S E C C C C
E C C E S S E C C C
E C C E S E S E E E

C 34 or 34%
E 38 or 38%
S 28 or 28%

where d is difference between x-values in two neighbouring fields and n' the number of such differences. The variance $s^2(p)$ is then obtained by multiplying the value given by equation (25) with the finite multiplier as above.

Approximate area estimates frequently accurate enough can be obtained without planimetric measurements. One way of approximating the areas is to insert a square-ruled network in the ocular and thus divide the field into small units (Fig. 15). If two kinds of tissues are present, the estimation is done by counting those small squares which are more than half-filled with the other kind of tissue. If the number of small squares in the network is 100, the approximate percentage is then directly obtained. When this method is used, an additional variance component is introduced due to rounding off in those small squares, where both kinds of tissue are present. This variance amounts to $\frac{mh^2}{12}$, where m is the number of small squares not completely covered with either kind of tissue and h the area of each small square. This variance is to be added to the variance due to area sampling between fields.

In practice, estimation of the variance in this way would be cumbersome, and other ways are given later for practical purposes. The above-given formulas are nevertheless of interest from the point of view of understanding the sampling process and of design of suitable sampling methods.

It may be good to mention already in this connection that the same network which just was found useful for making area estimates by examining the small sub-squares can also be used for making area estimates by point sampling (see page 67). In this case, the intersection points are examined. An intersection point which happens to be on, e.g., colloid, is denoted as a "hit" on colloid. Hits on the different tissue components are then separately counted, and the proportional area is accordingly estimated as in Fig. 15.

Line Sampling.

Estimation of relative areas in tissue sections is possible without any area measurements at all. Methods in which the area measurements are replaced by length measurements or by counts have long been used in agriculture and geography (*Yates*, 1949). Such estimation methods are fortunately equally applicable to the study of tissue sections.

Line sampling (*Uotila* and *Kannas*, 1952) is done by throwing lines over the section and by measuring the various intercept lengths as shown in Fig. 16. In practice this can be done on a projected image of the section or, automatically, with a scanning device (Fig. 17, see also page 82). The slide is continuously moved by a constantly running synchronous motor, whose revolutions are recorded

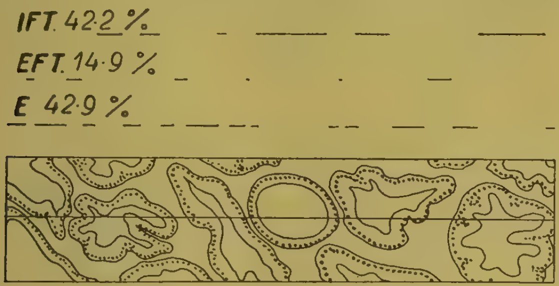


Fig. 16. Estimates of relative volume obtained with line scanning, IFT, intrafollicular tissue; EFT, extrafollicular tissue; E, Epithelium.

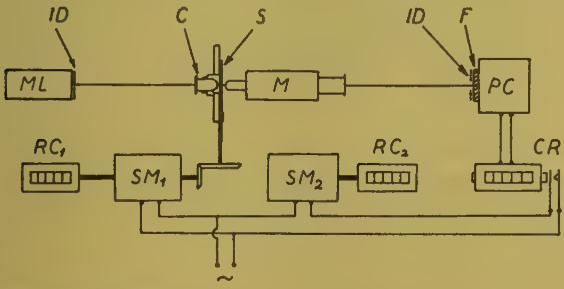


Fig. 17. A mechanical scanning device giving automatically the total length of the scanning line (the reading of the revolution counter, RC_1), the summed intercept length (the reading of the revolution counter, RC_2), and the number of intercepts (the reading of the counting relay, CR). C, condenser; F, filter; ID, iris diaphragm; M, microscope; ML, microscope lamp; S, slide; SM_1, synchronous motor moving the slide; SM_2, synchronous motor running when light is falling on the photocell, PC.

by means of a revolution counter. When one kind of tissue is stained and the rest of the section unstained, the intensity of light in a small area of the visual field indicates which of these two kinds of tissues is in this area. When the intensity of the transmitted light is high, i.e. when the unstained tissue is in the field, the phototube closes a relay and another synchronous motor will run. The total number of revolutions run by this motor is a measure of the length of the line passing through the unstained tissue and the number of revolutions run by the slide-moving motor gives the total length. When there are several kinds of tissues which can selectively be stained with different colours they can be differentiated in the same way by using several phototubes with suitable filtres (Fig. 18).

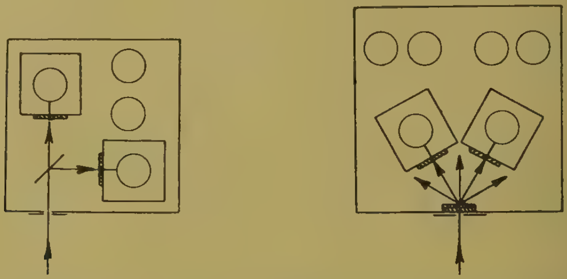


Fig. 18. Two methods for use in the scanning of three different kinds of tissue. In both, two phototubes are used, fitted with appropriate filters. In the arrangement on the left the light beam is divided into two beams by the use of a semireflecting mirror. In the arrangement on the right the light is diffused with an opal glass.

Let n be the number of lines on the section, x the summed intercept length and y the total length of each line. The relative area of the tissue over which the intercept lengths were measured is then estimated by

$$p = \frac{\sum x}{\sum y} = \frac{\bar{x}}{\bar{y}}. \tag{27}$$

The sampling variance of this estimate is obtained by replacing in formula (18) $\sum x$ for x and $\sum y$ for y. The following formula is thus obtained:

$$s^2(p) = \frac{p^2\left[\frac{s^2(x)}{\bar{x}^2} + \frac{s^2(y)}{\bar{y}^2} - \frac{2r_{xy}s(x)s(y)}{\bar{x}\bar{y}}\right]}{n}. \tag{28}$$

For actual estimation, $s^2(x)$, $s^2(y)$, and r_{xy} should be calculated from all values of x and y, when the lines are thrown over the section *purely at random.*

In line sampling, the relative variabilities of x and y often are nearly equal so that formula (20) applies. This formula shows the effect of large r. Generally, if the specific surface appears in islets small as compared with the length of the lines, r is likely to be near to unity, whereas if the surface appears in large bulks, the correlation is likely to remain small. Therefore, in the latter case more lines are needed than in the former.

If there are no great topographical differences between the different parts of the section, a crude estimate of the section variance can be quickly obtained by

(28a) $$s^2(p) = \frac{\sum (p' - p)^2}{n - 1},$$

where p' stands for $\frac{x}{y}$ for one line. This approximate formula presumes further that the length of the lines is nearly constant.

In *systematic line sampling*, in which parallel lines at equal intervals are used, the estimates of $s^2(x)$ and $s^2(y)$ should be calculated, not over all the section, but from differences between the values of x or y in subsequent lines, using formula (26) in the same way as in area sampling.

Point Sampling.

Measurements are completely replaced by counting in point sampling (*Chalkley*, 1943; *Lagerstedt*, 1949; *Attardi*, 1953). In purely randomized point sampling one point is fixed in the visual field by attaching a hair cross in the ocular. The visual field is then moved by setting each time randomly selected coordinates on the vernier scale of the slide holder (apparently randomized selection by moving the slide without any rule is subject to bias). When the relative volumes of, say, the nuclei, nucleoli, cytoplasm, blood vessels, etc. are to be estimated, the number of hits on these are counted. Let m out of n points thrown of the section hit on nuclei. The estimate of the relative volume of nuclear tissue is then

(29) $$p = \frac{m}{n}.$$

If the points are thrown purely at random the sampling variance of m is estimated by

$$s^2(m) = \frac{n^2 p(1-p)}{n-1}. \tag{30}$$

As n is conveniently kept constant, a simple formula is obtained for the variance of p:

$$s^2(p) = \frac{p(1-p)}{n-1}. \tag{31}$$

In *systematic point sampling* the situation is again different, the sampling variance of m tending to be smaller than that obtained from equation (30). In actual practice, however, formulas (30) and (31) can usually be applied for obtaining an approximation of the variances.

Systematic point sampling in a single visual field is conveniently accomplished by using the same square grid as that used for area estimation in Fig. 15, page 63. Hits at the 121 intersection points are then recorded, and an area estimate is obtained. Throwing several such grids on the section by moving the slide in a randomized way or at equal intervals results in a combined point and area sampling. While one component of the sampling variance, i.e. that in each field, obeys rules applying for point sampling, the other component, between fields, can be estimated with the formulas given for area sampling, (25) and (26), pages 61 and 62.

The practical value of the formulas above lies in that

a) they show how to calculate the standard error of an estimate, the standard error being the square root of the variance,

b) an investigation of the formulas reveals the main sources of variation thus enabling us to make an optimum or nearly optimum design of sampling and measurement.

In many practical situations the calculations involved in the above formulas will not be performed, because there are also other sources of variation present. Therefore, the formulas mainly serve as indicators as how to approach an optimum design.

Practical Estimation of Sampling Variance.

Above, a number of variance formulas were given. Some of them, especially those given in connection with systematic sampling, are not very attractive because of the amount of computational work. Therefore, other estimation methods may prove more economical and convenient in use. Indeed, in many cases it is likely that to *replicate* the samples is easier than to enter the cumbersome procedure of exhausting all information contained in one sample. For example, in line sampling the sample from one section consists of a number of constant-interval lines, differences between which can be used in variance estimation as described above. It may well happen that it is easier to take another sample (or three or more of them) on each section and record another series of sample estimates. The variability and thus the sampling variance can now be assessed exactly in the same way as described in connection with the error of method (see page 23). Simply, the error of method is to be considered identical with sampling variability. Thus, only the section estimates of relative area (or any characteristics) need to be recorded together with their duplicates, triplicates, etc. At first sight it might be thought that duplicates are not enough because only two "observations" on each section are obtained. However, a close study of the method of determining the error of method reveals that whenever the number of sections is not negligible, the error of method or, in this case, sampling variability can be estimated rather accurately. To take triplicates increases the precision considerably (*Fisher*, 1950).

Further information can be derived by recording the results for each line separately. If this is done, one obtains an idea of how the sampling system behaves statistically. In particular, it is then possible to make such alterations in the sampling methods that an optimum design is obtained: sometimes e.g. the intervals of the lines can be made larger without loss of accuracy, sometimes quite other designs may be indicated. Such studies, however, generally require close statistical control, whence it is advisable to consult experts. For details of applications, see *Snedecor* (1946), *Yates* (1949), and *Fisher* (1950).

A Warning for Systematic Sampling.

In spite of complications involved in calculating the random error, systematic sampling is generally to be preferred to pure random sampling. However, there is an important exception:

> If there is any periodicity in the material, systematic sampling should not be applied unless the method can be guaranteed to be independent of this effect.

For example, muscular tissue contains long largely parallel muscle fibres, and systematic line sampling is dangerous if the lines are parallel to the fibres. However, if the lines are put at an angle to the fibres, the effect is likely to be eliminated. Point sampling may be dangerous anyway in this case.

Subdivisions of Variance.

Above, the variance within sections was concerned. In actual practice, however, there are several sections to be investigated, and the aim is to obtain an estimate of, say, a gland. Let M be the number of a complete series of sections from the gland, m being the number of sections actually investigated. A gland estimate of the proportional area will then be based on these m sections so that

$$(32) \qquad p = \frac{\sum x_i}{\sum y_i}, \quad \left(\text{not } p = \frac{\sum \frac{x_i}{y_i}}{m}\,!\right)$$

the subscript referring to any section i. In estimating the variance of the gland estimate p, formula (18) (page 60) can again be applied, noting that

$$(33) \qquad s^2\left(\sum x_i\right) = m s^2(x_i),$$

where $s^2(x_i)$ can be estimated from the material as the variance between the section totals. Similarly for y. However, the m sections are a sample from a finite population of M sections, wherefore formula (33) is to be multiplied by the finite multiplier $\frac{M-m}{M-1}$. This holds for the case that the sections are a pure *random sample* from the totality of all sections. If they have been selected *systematically*, say every third section or so, then $s^2(x_i)$ should be estimated by means of differences of the totals of neighbouring sections as described in connections with formula (26), page 62.

Without going into the details (for which see e.g. *Yates*, 1949, and *Deming*, 1950), it must be pointed out that generally the variance of a gland estimate is composed of two terms, viz. one due to the average variance within sections (i.e. the average variance of the section estimates), and another due to the variance between the sections. The relative magnitude of these two effects in every particular case gives an idea of optimum design.

Finally, if a group of k animals have been treated as above, an estimate of the general relative area is required. It is obtained by adding both x's and y's over all animals and dividing. However, it must be emphasized that when the variances within animals are approximately equal—as usually is the case—it is more adequate to estimate the relative area by $\frac{\sum \frac{x}{y}}{k}$. The variance of such an estimate is again composed of two parts, viz. the variance within animals, i.e. the average animal variance, and the variance between animals. In many practical situations the quantitative techniques are accurate enough to keep the variance within animals small as compared with the variance between animals. In such case the variance can satisfactorily be estimated from the animal totals. The general formula (18) again applies. Note that no finite multiplier is introduced because the animals treated must be considered as a sample from a population of infinitely many animals.

Correction Formulas.

If a volume estimate has been done on the basis of the area projected on the plane of the slide, an error is introduced unless the borders between different tissues are perpendicular to the plane of the slide. This error can be avoided by using very thin sections or optics with a short depth of focus but it may be considerable when the relative volume of tissue particles is estimated in sections whose thickness is of the same magnitude as or exceeds the diameter of the particles. This is frequently the case when the relative volumes of particles such as nuclei and nucleoli are estimated. The values obtained can of course be used without correction and, as shown in *Lagerstedt*'s (1949) work, valuable results may still be obtained in comparative studies. Correction is nevertheless easily done and guards against false conclusions.

Consider a tissue section of thickness a containing spherical particles of radius r (Fig. 19). The area in the section plane covered by each whole particle in the section is then πr^2. Now, if a volume estimate is done by multiplying this area with a, a volume of $a\pi r^2$ is ascribed to each particle although the real volume is $\frac{4\pi r^3}{3}$. Similar volume is also estimated for a particle of which only one half is in the section. A gross error will obviously arise in thick sections. Such an error may lead to particularly distorted results when the relative volumes of large and small particles, say nuclei and nucleoli, are simultaneously estimated.

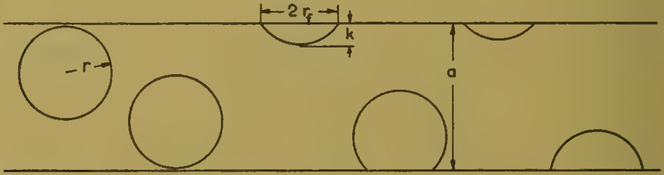


Fig. 19. Spherical particles with diameter r in a section with thickness a. The height of the smallest visible fragment is denoted with k, and the corresponding horizontal diameter with r_f. See text.

It can be shown that a correct estimate of relative volume is obtained by multiplying the estimate of relative projected area with a correction factor given below. If the specific projected area is A and the total area sampled A_0 then the real volume of the specific tissue in proportion to the total volume sampled V_0 is

$$\frac{V}{V_0} = \frac{4r}{4r + 3a} \cdot \frac{A}{A_0}. \tag{34}$$

This correction applies, however, only when all fragments of the particles can be recognized in the section. Especially in nuclear measurements this is not always the case, thin nuclear caps escaping notice. The following formula must then be used

$$\frac{V}{V_0} = \frac{4r^3}{4r^3 + 3ar^2 - 6rk^3 + 2k^2} \cdot \frac{A}{A_0}, \tag{35}$$

where k is the vertical height of the smallest fragment which can be observed. The value of k can be approximated by measuring the horizontal diameter ($2\,r_f$) of the smallest fragments visible in the section and applying the following equation

$$r^2 = (r - k)^2 + r_f^2. \tag{36}$$

As it is difficult to know what is the smallest fragment still visible, k is better estimated by a method to be described later (page 77). In strongly stained small particles k is small and can be neglected. When comparative measurements are done, formula (35) may mostly be replaced by formula (34).

CHAPTER VII

Counting, Sizing and Various Quantitative Criteria

Introduction.

Estimation of relative volumes as described in the preceding chapter is certainly in many cases useful for assessing the state of a gland or organ. Nevertheless, many other characteristics can be measured which may prove equally or even more valuable indicators.

When the relative volume of a tissue is shown to increase during an experiment, it cannot be known whether this relative increase is connected with an absolute volume increase. Consider, e.g., the pancreatic islets. A relative increase in the volume of the islet tissue will occur not only when the absolute volume of the islet tissue increases, that of the exocrine part being unchanged, but also when the absolute volume of the islet tissue is unchanged but the exocrine tissue atrophies. The biological meaning of the observed change is distinctly different in these two cases. They can be differentiated by estimating the total volume, which for all practical purposes is sufficiently accurately done by weighing the gland. The absolute volume can then be estimated. Errors due to different specific gravities in the two kinds of tissue are very unlikely to affect significantly the estimates of *changes* in the absolute volume.

Even if it thus can be seen whether the absolute volume of the endocrine tissue has increased, decreased or kept constant during the experiment, other information may be needed. An increase in the volume, e.g., may be due to an increase in the size of individual islets, to an increase in the number of islets, or to both. Further, some islets may increase their volume more than the others, and the form of the islets may be altered. The interpretation of the recorded changes may be essentially influenced by factors like these.

Similar considerations apply to individual cells. Ratio of the volume of the nuclear protoplasm to the volume of the cytoplasm is without doubt a valuable measure but much additional information can be obtained by measuring the volume of individual nuclei and by counting the number of nuclei in a unit volume of tissue. In this way, it is possible not only to analyze the size-frequency distribution of the nuclei but also to obtain another estimate of the relative volume of the nuclear tissue.

Counting in Diluted Homogenates.

Counting of erythrocytes is one of the earliest applications of quantitative methods on histology. As is well known, it is done by diluting a small sample of blood in known proportion with some isotonic fluid and by counting the number of cells per unit area in a chamber of known depth. Exactly the same method can be used for counting other cells. As the dilution, the thickness of the layer of diluted blood in the chamber and the area in which the cells were counted are known, the number of cells in cu.mm. of blood is easily calculated.

The same method can be applied also to firm tissues. A piece of tissue is first weighed. It is then homogenized in some suitable fluid, e.g. in M/2 sucrose solution. The homogenate is diluted to a known volume, thoroughly mixed, and the number of, say, nuclei is counted in the normal way in the counting chamber. As mentioned earlier (page 37), the homogenate can be fractioned by differential centrifugation. By isolating pure fractions of nuclei, of various types of cytoplasmic granules, etc. chemical analysis of each constituent can be done conjointly with counting. Moreover, the homogenate can be used for measuring the size or stainability of individual particles.

Poisson distribution has been observed to apply to counts obtained in a haemocytometer (*Fisher*, 1950) and the variance is therefore equal to the number of counts. It is mostly preferable to count the number of particles in several square areas filling the field of vision and large enough to include some 50 to 100 particles per area. To avoid counting twice the same particle this area should be divided into 16 (4 × 4) to 100 (10 × 10) smaller squares. When the number of particles is large per selected unit of area this number is distributed nearly normally (see page 15). As the whole count can be thought to be composed of several sub-counts distributed according to Poisson, it is nevertheless justified to estimate the variance equal to the total count. Suppose that the number of, say, nuclei in 5 squares, each divided into 100 sub-squares, is 2125. The variance then is also 2125 and the standard deviation $\sqrt{2125} = 46 = 2.2\%$. It is wise to determine the actual standard deviation by double counts (see page 23). If it deviates much from 2%, something is wrong in the counting system. By counting the number of particles in each small square one may actually test whether the Poisson distribution applies, which may be a useful check when nonhomogeneity in the particle distribution can be expected to interfere.

The crucial factors in the counting are the homogeneity of the sample and the accuracy of the dilution. The depth of the layer of fluid in the counting chamber must naturally be strictly constant also. The square ruling may be either in the slide itself, as is usual in the haemocytometer slides, or it may be inserted in the microscope ocular. Manually operated mechanical tally counters are of great help in all counting operations. For possibilities of approximation of particle number with photometric measurements, see page 95.

Counting in Tissue Sections.

Measurements in sections allow a close study of the topographic relations between different cells and cell constituents, which is not possible in homogenates. Furthermore, not all tissue units survive the homogenization. Histological specimens must be used in such instances, e.g. in estimating the number of particles such as glomeruli in the kidney or *Langerhans* islets in the pancreas.

On the other hand, besides whole particles their fragments are present in tissue sections. The number of the fragments in proportion to the number of whole particles increases when the section thickness decreases. When the tissue thickness is lower than the diameter of the particles only fragments are present. The actual number of particles per unit volume is thus always smaller than the number obtained from sections. In case the particles are randomly distributed and spherical the following formula can be used to obtain the correct number (*Floderus*, 1944):

$$N = \frac{an}{a + 2r - 2k} \tag{37}$$

where N is the real number of particles in each cu.mm. of tissue, a the section thickness (μ), r the radius of the particles (μ), k the vertical height of the smallest fragment which can be observed (μ), and n the counted number of particles per each cu.mm. of tissue.

Formula (37) can also be written in another form

$$N = \frac{n'}{a + 2r - 2k}, \tag{38}$$

where n' is the number of particles per 1000 sq.mm. tissue counted. The value of k is best approximated by making counts in sections of different (known) thickness and by setting $\frac{n'_1}{a_1 + 2r - 2k} = \frac{n'_2}{a_2 + 2r - 2k}$. (See also formula (36), page 73). Some interesting facts can be seen from formula (38). First, when a is large as compared to $2r$, N can be approximated by $\frac{n'}{a}$, i.e. the fragments can be neglected. On the other hand, supposing that a is constant and that r is subject to variation, the error in estimating N due to this variation sharply increases, if the ratio $\frac{2r}{a}$ is increased above 1. This could seemingly be taken to indicate that counts should always be done on as thick sections as possible (the section thickness cannot, of course, be increased so much that the particles will be in two layers). However, this applies only, if the section thickness can be accurately controlled. If this is not the case, and the particle diameter is fairly constant, it is better to do the counting in sections thin as compared to the particle diameter. This can be illustrated with a simple example.

Suppose that spherical particles of uniform diameter 20 μ and their fragments are counted in a section 100 μ thick, the value of k beeing 2μ. Let the total number of counts per 5 sq.mm. be 1000. Then the real number of particles per cu.mm. of tissue will be $\frac{n'}{100 + 20 - 4}$, or, as $n' = \frac{1000 \times 1000}{5}$, $N = \frac{200\,000}{116} \sim 1724$. Now suppose that the section thickness had been erroneously estimated 20 % below the real value, at 80 μ. Then, the estimate of N will be $= \frac{200\,000}{96} \sim 2083$ or some 21 % above the true value.

Let us then do the counting in a section 5 μ thick. Then the total count in 50 fields, each of 1 sq.mm., would be, say, 1810. Then $N = \frac{1810 \times 1000}{50(5+20-4)} \sim 1724$. Let the section thickness be falsely estimated at 20 % below the true value. Then N is obtained $= \frac{1810 \times 1000}{50(4+20-4)} =$ 1810 or only some 5 % above the true value. Even if a is put at 50 % of the true value, the calculated value of N is 1957 or only 14 % higher than the true number. This is the more interesting as in a homogenate where no fragment error is present, a 20 % error in the specimen thickness always causes a 20 % error in the count.

Thus, selection of the tissue thickness must in each case be done separately with due regard to the specific circumstances. When high magnifications are used, it is good to remember that the effective thickness of a section can never exceed the depth of focus of the optics used, no matter how thick the section is. This fact may be used to eliminate the error due to variations in the section thickness.

Estimation of Particle Size.

The *size* of particles can equally well be measured in diluted homogenates, in smears, and in sections. Indeed, size measurements are necessary in sections before correct counts can be calculated according to formulas (37) or (38). The volume can be estimated from the diameter, if the particle is nearly spherical. In homogenates of sufficient thickness to allow the particles retain their spherical form, and in sections the volume is obtained as $\frac{\pi d^3}{6}$. In smears the true volume of a particle—flattened in the smear—is proportional to something between the 2nd and 3rd power of the diameter. The volume of elongated particles can be approximated by measuring two diameters (say a and b) perpendicularly to each other and by estimating the volume at $\frac{\pi a b^{3/2}}{6}$. Size measurements in elongated particles are, however, subject to errors difficult to assess, as only one projection of the particle in one plane is available for examination. For instance, the volume of a rotation ellipsoid with respective diameters

10 and 20 μ is $= \frac{4\pi 5^2 10}{3} \sim 1000$ cu.μ. When the long axis of such ellipsoid is perpendicular to the plane of the section, the volume is estimated at $\frac{\pi 10^3}{6} \sim 500$ cu.μ. Therefore, there is little sense in applying different specific correction formulas for the estimation of the volume of tissue particles of different form in sections or homogenates unless their orientation is taken into consideration. The error discussed can be diminished by measuring only particles whose long axis is in the plane of the section (see *Timonen*, 1950).

The diameters of the particles should be measured in sections of about the same thickness as or thicker than the particles. This is necessary, because reliable information of the particle size and orientation can be obtained only from particles whose centre is in the section. That the center is in the section can be made sure by elevating and lowering the microscope objective. If the diameter of the particle first increases and then decreases, the center is in the section. The largest cross-section of each particle is then measured.

Measurement of particle diameters by means of an ocular micrometer is usually cumbersome. It is more convenient to draw the magnified image of the largest cross-section of each particle as described earlier (page 54). Alternatively, direct measurements can be carried out on a projected image. For statistical analysis, the size-frequency distribution is studied along the lines drawn in chapters I and XI. If the class limits can be given before the measurements, time can be considerably saved by using a reference series of circles of different sizes, representing the class limits, and by simply counting the number of particles falling into each size class. This method has been used already in 1889 by *Malassez*. The number of classes required is dependent on the form of the distribution curve, curves with two or more tops needing a relatively large number of classes. This, on the other hand, makes a large number of observations necessary (see page 12). If the particle size is known to be e.g. lognormally distributed, a quick approximation of the size can be obtained by measuring the largest particle and the smallest particle of the sample and by estimating the total number of particles that could have been measured (see *Leigh-Dugmore*, 1954).

Randomization is urgently necessary in selecting the particles to be measured. Although a subjective step is always necessarily included in the selection of particles with the center in the section, bias is avoided by moving the slide after a random start (see page 18) at equal intervals. Every particle should be characterized by a uniquely determined point, e.g. the "most northern" point. If the characteristic point of a particle falls within a defined area in the visual field, the particle is included in the sample. If the center of a particle selected in this way is not in the section, the slide is moved again. When measurements are done on both spherical and elongated particles, a bias is easily introduced, as more elongated particles must be rejected than spherical ones. Such an error can be corrected if the ratio (longest axis/shortest axis) of each particle is measured.

When a size-frequency distribution curve based on a sufficient number of measurements is available and the number of particles per unit volume of tissue has been estimated as described earlier in this chapter, an estimate of the relative volume of the particle tissue is obtained. It is useful to compare this estimate to another one obtained as described in chapter VI. Thus, overlooked sources of error can be detected.

Simultaneous Counting and Sizing of Particles.

Timbrell (1952) has described an ingenious method for simultaneous measuring and counting of microscopic particles. The image of the microscopic specimen is made to oscillate by means of a mirror. Light is allowed to pass through only when the image is in either of the extreme ends of its travel. Particles with a diameter smaller than the oscillation amplitude are then shown as double spots, while the images of particles with a diameter larger than the oscillation amplitude overlap. This overlapping area is dark, and by counting the number of dark areas at a given amplitude the number of particles with a diameter larger than this amplitude is obtained. By repeating the procedure at different amplitudes a cumulative size-frequency curve can be designed.

Fig. 20 shows an apparatus which can be used for the purpose. It is essentially that described by *Atherton* (1953) for determinations of particle sizes in photographic negatives. The light from the microscope (M) is passing through a disk (CD) on an aluminized mirror (AM), and the image of the specimen is projected on the screen (S). An electric motor (EM) rotates the disc and the cams (C) in a rotary shaft controlling a loudspeaker coil (LC) which in turn moves the mirror. One half of the disk is coloured red, the other one green.

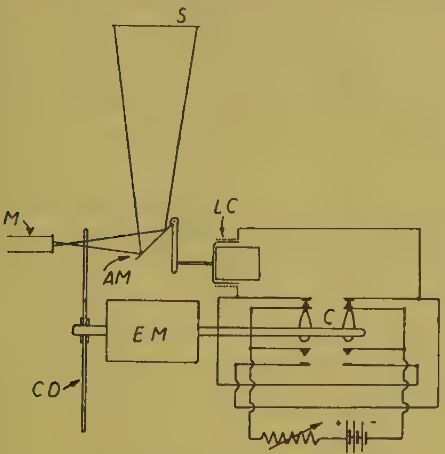


Fig. 20. A device for counting and sizing. For use see the text. AM, aluminized mirror; C, contactors; CD, colored disk; EM, electric motor; LC, loudspeaker coil; M, microscope; S, screen.

The rotary switch generates a square wave so that the mirror is at the one end when the light passes the green half and at the other end when the light passes the red half of the coloured disk. Particles which transmit light in the negative appear red at one end, green at the other and the overlap is bright yellow. When counts are done on microscopic specimens with light-*absorbing* particles, the disk can be omitted. The amplitude of the mirror movements is controlled by means of a rheostat in series with a battery.

The described method is particularly serviceable in sizing and counting of strongly stained small particles. The actual counting can be done visually with much less labour and eye strain than with the methods described earlier in this chapter. Using this principle, automatic counting by means of properly designed electronic phototube-magnifier-filter-counter circuits is also possible.

Automatic Scanning Methods.

The problem of measuring the number and size of microscopic particles is not limited to biology. It arises also in numerous branches of industry, in geology, etc. As visual counting methods are tedious and subject to bias, several groups of investigators in different fields have contributed to research for finding adequate automatic counting methods. *Walton* (1952) has reviewed recent results obtained in such studies in Great Britain. Several ingenious devices have been designed capable of automatically giving the size-frequency distribution of particles. All of these counters are complicated and most of them make use of the recent advances in electronics. Still, the underlying principle is simple in most of them.

The specimen is scanned with a light spot moving progressively over the field or of a representative part of it. The changes in the intensity of light caused by the particles, or the intercept lengths, are recorded. Thus, the basic principle is related to that of the line sampling, and any of these apparatus can without difficulties estimate automatically the relative area covered by particles. Difficulties arise in the estimation of particle number and size. If the scanning line is broader than individual particles, two particles close to each other may be counted one. If, on the other hand, the scanning lines are very thin and close to each other, two adjacent lines may hit on the same particle, and falsely high number of particles is obtained. The errors thus arising have been compensated for by the simultaneous use of two scanning systems in compensatory circuits or by electronic "memory systems" (see also *Hodkinson*, 1953; *Jordanides* and *Chamberlain*, 1954).

Different arrangements are possible for moving the scanning spot in relation to the specimen. The specimen itself may be moved, as in the apparatus shown in Fig. 17, or the light beam is moved, either mechanically as in the scanning device by *Mellors* and *Silver* (1951) illustrated in Fig. 21 or by using a flying spot in the film-scanning oscilloscope tube as a source of light (*Young* and *Roberts*, 1951). A further alternative is to use a television camera in which the image of the specimen is scanned by an electron beam. In most cases a photomultiplier cell is a suitable detector of light (see page 130).

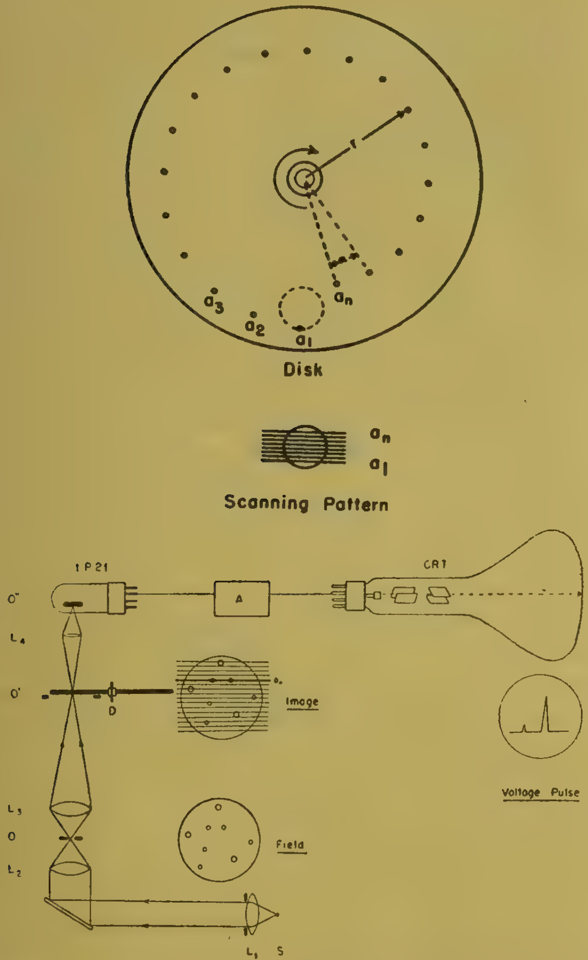


Fig. 21. Mechanical scanner designed by *Mellors* and *Silver* (1951). S, source of Köhler illumination; L_1, field lens; L_2, condenser; O, object; L_3, objective; O′, image; D, scanning disk; L_4, auxiliary lens; 1 P 21, photomultiplier; A, amplifier; CRT, cathode-ray tube, with voltage pulse from cells scanned along line a_n.

Various Quantitative Criteria.

The intercept lengths in line scanning give valuable information about the specimen. When certain conditions are fulfilled, e.g. when the specimen is composed of spherical particles distributed at random over the whole area, the intercept-length distribution can be used for calculating the size-frequency distribution (*Hawksley*, 1951). When the particles are variable of shape and they are not randomly distributed, other methods must be used for obtaining the number and size of the particles, as the distribution of the intercept lengths is essentially influenced by the form and distribution of particles. Use can be made of this fact in the converse sense.

Thus, if some tissue is very irregularly distributed, say, in a gland, it may be difficult to describe its structure in an adequate way. By scanning the tissue with parallel lines and by studying the distribution of the intercept lengths, an empirical *line criterion* is obtained of the topographic pattern which enables quantitative comparison between two glands. Useful information can be obtained in this way, although the data derived may seem to have little biological meaning. Actually this kind of criterion is dependent on many properties of the tissue at the same time and may therefore prove sometimes even more sensitive indicator of experimentally induced changes than some "more sensible" criterion.

An *area criterion* can naturally be obtained in an analogous way. Grids of equal size are thrown over the specimen at equal intervals and the areas of the specific tissue are measured in each grid. Distribution of these areas can then be used as an empirical indicator of the state of the organ. As the grid size is one parameter in the distribution, it must be kept constant in comparative studies.

Both these criteria are one kind of *metameters* of the properties of the specific tissue. The use of such metameters can equally well be applied to individual particles. Thus, instead of measuring the size accurately (i.e. by planimetric measurements of a complete series of thin sections) *size metameters* can be used. A few size metameters are enlisted:

a) largest diameter of the particle,
b) mean diameter of the particle,
c) diameter of the particle in a given direction,
d) product of two orthogonal diameters,
e) area of the smallest circle drawn outside the particle,
f) circumference of the particle,
g) area of the largest circle drawn inside the particle.

The above metameters refer to the area of the particle projected on the plane of the microscopic slide. If the particle is a sphere, all of these metameters uniquely define the size of the particle but if the shape of the particle is non-spherical, one of these metameters is in each individual case better indicator of size than the others. Thus, the *form* of the particle determines the choice of the metameter.

This fact may be utilized in aiming at quantification of the form. Several *form metameters* can be used in this sense. To list a few:

a) ratio maximum/minimum diameters,
b) ratio outside circle radius/inside circle radius,
c) ratio circumference/area,
d) ratio surface area/volume,
e) number of "gulfs".

Of these criteria a) and b) may be useful, when the particles are ellipsoidical, in which case the others of the mentioned criteria are of little use. On the other hand, criteria c), d), and e) can be informative when the shape of the particle is very irregular. The irregularity of a particle may be a biologically important measure, the ratio surface area/volume being of great significance in the transfer of substances from one tissue to another. An example of the use of the number of gulfs as a criterion is given later (page 147).

The study of certain *distances* may sometimes be of interest. Thus it is well known that the position of the nucleolus in the nucleus of a nerve cell tends to acquire an excentric position when the cell is active (see e.g. *Hydén*, 1943; *Eränkö*, 1951). The position of a nucleolus in a nucleus can be defined by an excentricity ratio. Let R be the radius of the nucleus, r the radius of the nucleolus, and a the distance of the centre of the nucleolus from the centre of the nucleus (Fig. 22). The excentricity ratio is then

$$E = \frac{a}{R}. \tag{39}$$

Maximum value of E is, of course, $\frac{R-r}{R}$. Now, the line between the two centres is not always in the plane of the slide, wherefore the nucleolus may in some cells seem in the microscope to be in the

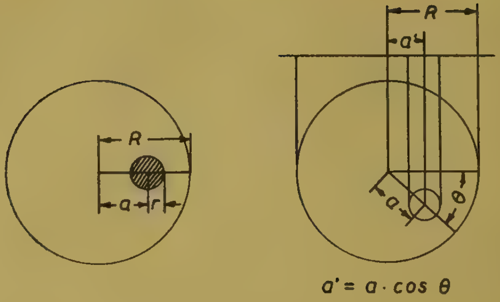


Fig. 22. See text.

centre of the nucleus, although all nucleoli would actually be attached to the nuclear membrane. Let a' be the horizontal distance between the centres of the nucleus and the nucleolus. Then $a' = a \cos \varphi$, where φ is the angle shown in Fig. 22. Then the mean true excentricity ratio can be shown to be

$$E = \frac{\pi \sum \frac{a'}{R}}{2n}, \tag{40}$$

where n is the number of observations.

Several other morphological properties of tissues could be exemplified in which quantification is possible, such as orientation of fibres or particles, lengths of secretory tubuli, etc. Once the quantitative approach is accepted, optimal ways of quantification can be found in each specific problem without difficulties. We will therefore avoid serving the old wine in a new bottle again and turn to a new aspect, the light absorption of tissues.

CHAPTER VIII

Absorption Measurements

General Principles.

Principles of absorption photometry, as developed by chemical investigations, can be applied to quantitative microscopy of tissues. Pertinent handbooks such as that by *Lothian* (1949) and that by *Mellon* (1950) may be consulted by the interested reader for fuller information.

Quantitative spectrophotometry is made possible by the fact that the proportion of light absorbed by a solution is dependent on the concentration of the absorbing substance. The concentration can accordingly be estimated by measuring the magnitude of the absorption.

Besides the concentration of the light-absorbing material many other factors influence the amount of light absorbed by the solution. These factors are

a) chemical composition of the substance,
b) wavelength of the light used,
c) thickness of the absorbing layer,
d) monochromacy of the light used (width of the waveband),
e) light-diffusing properties of the medium, and
f) homogeneity of the absorbing substances in the medium.

In chemical analysis the solutions to be measured are usually clear. Furthermore the substance in solution is always evenly distributed in the medium. Thus, problems due to light refraction and uneven distribution of the absorbing substances are avoided. This is rarely the case in tissue sections. In other respects, knowledge accumulated by chemical investigations can be directly applied to quantitative analysis of tissue sections. In the following treatment of the subject, factors influencing the light absorption in clear solutions will first be discussed.

Lambert's Law.

The proportion of light absorbed by an absorbing layer is not influenced by the intensity of incident radiation. This is Lambert's law, and it can be expressed by the following equation

$$\frac{I}{I_0} = \text{constant}, \tag{41}$$

where I_0 is the intensity of light entering and I the intensity of light transmitted by the absorbing medium. Let μ be a constant depending on the absorbing properties of the substance and l the thickness of the absorbing layer. Then the constant in equation (41) can be replaced with $e^{-\mu l}$, where e is the base of the natural logarithm. The equation (41) can now be expressed in the form

$$I = I_0 e^{-\mu l} \quad \text{or} \quad I = I_0 10^{-\gamma l}, \tag{42}$$

where γ is another constant. This law has been observed to hold true in practice if monochromatic radiation is used.

Beer's Law.

Beer's law claims that the magnitude of absorption is a function of the number of absorbing molecules only, dissolved in a non-absorbent solvent. It can be expressed in the following equation

$$I = I_0\, 10^{-Ecl} \quad \text{or} \quad \log\frac{I}{I_0} = -Ecl, \tag{43}$$

where E is so-called extinction coefficient, c the concentration of the substance in the solution, and l the thickness of the layer through which the light passes. $\frac{I}{I_0}$ is commonly called transmission (t) of the light and light loss is expressed either in terms of transmission or in terms of extinction $d = -\log t$, also called density. Then the equation (43) gets the form

$$d = Ecl \quad \text{or} \quad c = \frac{d}{El}. \tag{44}$$

As E is a constant the magnitude of which depends on the absorbing substance, concentration of this substance can be estimated by measuring the extinction caused by a solution having a depth l. The value of E is first to be estimated by measurements in solutions of known concentrations.

l is usually given in cm. and c in %. Correspondingly E is given in terms of % and cm., e.g., $E^{2\%}_{5\,\mathrm{cm.}}$ for each wavelength.

Beer's law is frequently valid and it has been extensively used in quantitative spectrophotometry. However, deviations from it are not few, wherefore the validity must always be checked up empirically before application.

Deviations from Beer's Law in Clear Solutions.

Many coloured substances absorb less light at *high concentrations* than could be expected if Beer's law were valid. Numerous such examples can be given among dyestuffs commonly used in histological staining techniques. To mention one example, thionin, whose absorption maximum in dilute solutions is at about 6000 Å, shows a decrease in the molar extinction to one half, when the concentration is increased from 0.0025 M to 0.025 M. This decrease in the absorbing properties of thionin at this wavelength is accompanied by a pronounced change in the form of the absorption spectrum, which manifests itself in a change of the colour of the solution.

Changes in the light-absorbing properties of substances due to changes in their concentration can be ascribed to causes such as changes in the ionization of the substances, reversible polymerization, changes in the refractive index of the solution, etc. These deviations are likely to be present to a pronounced degree in stained sections, where concentrations of dyes are high.

In addition, the light extinction may be affected by *reciprocal interaction* with substances which in themselves do not absorb light at the same wavelength, e. g. with proteins, present in high concentrations in animal tissues. This fact in mind it may be evident that, without direct evidence of the validity, Beer's law should never be expected to hold true in stained sections.

Beer's law applies further with *monochromatic light* only. All coloured substances have a characteristic absorption spectrum, i.e., the absorption varies with wavelength. When the concentration of an absorbing substance is increased without altering the depth of the absorbing layer, the extinction increases proportionally at each wavelength. However, as the corresponding transmission changes are different at each wavelength because of differences in the extinction coefficients, the total extinction over the whole wavelength range does not show an increase in the same proportion as the concentration. This *waveband error* can theoretically be avoided by working with a constant value of $l\,c$. This is hardly possible in tissue photometry, however.

Errors due to Refractive-Index Differences.

In a microscopic specimen of biological material, special difficulties appear because of refractive-index differences in tissues and cells. These difficulties and their elimination in microspectrographic work have been extensively discussed by the pioneer in this field, *Caspersson* (1950), whose recent monograph is an invaluable guide to everybody interested in photometry of tissue sections. The reader will also find a critical article by *Glick*, *Engström*, and *Malmström* (1951) interesting in this respect.

The effect of light refraction and reflection is best illustrated by an example. Consider a plane parallel cuvette filled with undiluted blood, the thickness of the cuvette being about 0.5 mm., which is enough to assure an even light loss over the whole area. When a parallel beam of light rays with a wavelength of about 4100 Å is allowed to pass through the cuvette, it will be seen that the intensity of light transmitted is very small in comparison to the intensity of light entering the cuvette. This can be understood knowing that haemoglobin contained in the erythrocytes shows a strong absorption at this wavelength. This is not the whole story, however.

If the transmission measurement is repeated at a wavelength of ca. 7000 Å, where the light absorption by haemoglobin is negligible in comparison to that at 4100 Å, it is found that the blood causes a marked reduction in the intensity of the transmitted light. If a small amount of some haemolytic agent is now mixed with the blood and the transmission is measured again at the same wavelength, the transmission is near 100 per cent, although no chemical change is responsible. This difference in the transmissions of intact and hemolyzed blood is due to refraction and reflection of light by erythrocytes, whose refractive index is higher than that of the plasma. A similar loss in the intensity of the transmitted light can be demonstrated with completely transparent glass spheres immersed in water. Any particles with a refractive index higher than that of the surrounding medium cause a deviation in the direction of the originally parallel light rays, and unless all these deviated rays are hitting the light-sensitive surface of the measuring instrument an apparent absorption occurs.

Elimination of Scatter Errors.

As *Barer* (1952 g) has demonstrated, spectrophotometric absorption measurements on erythrocytes can also be done with intact cells without the disturbing influence of light refraction. By immersion of the red blood cells in an albumin solution whose refractive index is equal to the average refractive index of the erythrocytes, light loss due to reflection and refraction is small enough to allow detailed analysis of the absorbing properties of living cells. *Barer* (1953 c) made the very interesting observation that the absorption spectrum of erythrocytes as estimated in this way essentially differs from that of hemolyzed blood.

The angular intensity distribution of light behind the specimen is dependent on the size of the refractile particles, on the wavelength of the light, and on the magnitude and sharpness of the refractive-index difference between the surrounding medium and the particle. The light scatter tends to increase with decreasing wavelength and with diminishing size of the refractive particles. Factors determining the scatter are, however, complex and difficult to predict. Selective loss of light because of scatter at some particular wavelength is possible although the responsible particles were as such completely transparent at this wavelength. Difficulties due to light scatter are large in the ultraviolet part of the spectrum. In visible range the scatter is usually small and, in most cases, it can be further diminished by the use of appropriate mounting media. Proper optical conditions are nevertheless obligatory even in the visible range.

In an ingenious way *Caspersson* (l.c.) was able to produce a gradual change in the refractive index by mounting the freeze-dried section in anhydrous glycerol, in which a gradual swelling of proteins at the boundary smoothens the jump in the refractive index. Even so, the specimens scattered parallel light rays in so far that 5 % of the light was refracted more than 30° from the optical axis. The condenser aperture was in actual absorption studies chosen near 10°. To be able to collect 95 % of the transmitted light, the angular opening of the lens must thus be 2 × 40°, corresponding to a numeric aperture of 0.85.

Another way to eliminate the scatter error is to use diffuse light instead of parallel light rays for the illumination of the light-scattering surface. This method is much used in the densitometry of photographic plates. As the light rays enter evenly from all directions below the object plane, disturbing images due to refractive-index differences disappear and the intensity distribution in different parts of the image formed by a lens corresponds to that in the object. This method can be conveniently substantiated also in microscopic work by inserting an opal glass immediately below the section, preferably by mounting the section directly on the opal-glass surface, in which case the light loss is small. At least in visible light the results seem promising. Actually this method is to a certain degree opposite to that used by *Caspersson*. In his technique the specimen is illuminated with nearly parallel light rays and the transmitted light is collected in an objective with a large aperture, while in the last-described way the illuminating aperture is large in comparison to the objective aperture. Usefulness of both these methods in the visual range can be easily compared by the reader. The advantages for some purposes of an opal glass can be demonstrated by examining fairly thick sections (say 20 μ) mounted in glycerol at relatively low magnifications (ca. 300 ×). In thick sections a part of the section is below and a part above the sharply focused plane, and if the illuminating aperture is smaller than, or even equal to, the objective aperture, the image is blurred by refraction images from particles in out-of-focus planes. Though the use of an opal glass is therefore sometimes preferable to Köhler's illumination in work with thick sections and low-aperture objectives—and this is the case particularly in photomicrography—it should be realized that accurate photometric measurements in small areas of a single cell can only be done in thin sections and with high-aperture objectives, in which case *Caspersson*'s arrangement is better.

Other Sources of Error.

Further complications in microspectrophotometry may arise because of orientation of molecules (see *Commoner*, 1949; *Commoner* and *Lipkin*, 1949) and of anomalous dispersion. As these factors are not frequent and as they cause difficulties mainly in the ultraviolet region, the reader is referred to *Caspersson*'s monograph and to further references given there.

Contact Photomicrographs.

In this connection it is suitable to mention that photographs which can be used for spectrophotometric purposes can be prepared simply by making a contact copy of the specimen on a high-resolution plate. An analogous technique has been successfully used for several years for studying the distribution of radioactive substances (radioautography) and of radio-opaque materials (microradiography) in tissue sections. To our knowledge the technique has not previously been extended to the spectral area of light rays but this can equally well be done when proper conditions are fulfilled. To avoid complications due to light refraction, diffuse light is used, just as in the ordinary procedure for making contact copies. To be able to resolve small cell constituents, the section must be very thin (preferably 1 μ or less) and it must be at close contact with the photosensitive emulsion (plates without gelatin over the emulsion must be used). The section is preferably attached on the middle of a quartz glass, on which it is, if necessary, deparaffinized, brought to water, coated with a thin layer of celloidin and allowed to dry. The dry section is tightly pressed on the plate and exposed in the visible range through an opal glass and in the ultraviolet region through two ground quartz glasses. By exposing under standard conditions with monochromatic light at different wavelengths, an idea of the absorption spectrum of different tissue and cell constituents can be obtained without expensive optics otherwise necessary for work in the ultraviolet. Beckman's photometer may be used as a source of monochromatic ultraviolet light.

Uneven Distribution of Absorbing Materials.

In histological sections, absorbing materials are rarely evenly distributed over the section, and if they are, the use of microphotometric methods is mostly of little significance. The main advantage of these methods is indeed that they make possible quantitative estimations of very small amounts of substances in small tissue constituents. Unless the measurements are carried out on a restricted part of the specimen, within which the distribution of the absorbing materials is even, special precautions must be taken to obtain correct results.

Let us return to the example of blood photometry again. If the thickness of the cuvette is now diminished to 2 μ, and the transmission measurement is repeated at 4100 Å, the extinction observed should be 2/500 that obtained with a cuvette thickness of 0.5 mm., according to Lambert's law. In practice, however, it will be found that the actual extinction is far smaller than the expected value, even if the measurements are carried out after elimination of refraction effects and with very monochromatic light. The reason for the unexpectedly increased transmission is simply the fact that the cuvette has been made so thin that the light is readily transmitted through plasma areas not covered with erythrocytes. Depending on the ratio (total area covered by erythrocytes/total area across the cuvette) variable deviation from Lambert's law is obtained. Although the erythrocytes were completely opaque, in which case a transmission of zero would be obtained with any cuvette thickness exceeding that necessary to prevent the appearance of "plasma holes", light would be transmitted with thicknesses below this critical value. Indeed the transmission would then be an accurate measure of the relative area covered by erythrocytes (cf. chapter VI). With haemolyzed blood, of course, no such deviations would be seen.

By simply applying Beer's law an idea is obtained of the quantitative significance of this phenomenon. This has been done earlier by *Glick et al.* (1951) who calculated the error in the optical density due to nonhomogeneity under various conditions. The following treatment approaches the same problem from a somewhat different angle.

Consider a layer of blood of the same thickness l as that of one erythrocyte. Suppose that each erythrocyte is an equally high flat cylinder and that the haemoglobin concentration c is equal in each cell. Let E be the extinction coefficient of haemoglobin, the area on which the transmission is measured being 1 and a—in this case always < 1—the total area covered with erythrocytes. Then, according to equation (43) the intensity of light passing through the erythrocytes is

$$I_1 = aI_0 10^{-Ecl} \tag{45}$$

and that of the light transmitted through cell-free areas with no absorption

$$I_2 = (1 - a) I_0 . \tag{46}$$

The total intensity transmitted is

$$I = I_1 + I_2 = I_0 (1 - a + a\,10^{-Ecl}) . \tag{47}$$

In haemolyzed blood, on the other hand,

$$I' = I_0\,10^{-Eacl} . \tag{48}$$

It will be apparent that $I \neq I'$. If $\frac{I}{I_0}$ and $\frac{I'}{I_0}$ have been measured, a can be calculated from equations (47) and (48). If further the average area of the erythrocytes is known, their number in the field can be approximated by two transmission measurements, an application which may prove useful in some instances (cf. page 75).

If a and $\frac{I}{I_0}$ are known, it is possible to calculate c applying the following equation, derived from equation (47):

$$E\,c\,l = \log \frac{a}{t + a - 1} \tag{49}$$

where $t = \frac{I}{I_0}$. Note that c then is the true concentration in the absorbing elements. The mean concentration of the whole specimen is obtained by multiplying c with a. Fig. 23 shows the dependence of Ecl on t at different values of a. It will be seen that Ecl sharply increases with decreasing t at low values of a, which necessitates accurate measurement of t to avoid gross errors.

In sections of biological material the absorbing substances are frequently present in widely varying concentrations in different parts of the section, the individual extinctions ranging from zero to high values. In such instances the above theoretical considerations are of little help if it is wanted to know something about concentrations. Transmission measurements may even in such case prove useful, if their limitations and the discussed sources of error are taken into consideration. In many experimental conditions the changes are large enough to be detected with inaccurate methods. It is, however, preferable to try to get rid of the nonhomogeneity within the field to be measured. As mentioned earlier, this can be done by performing the measurements in *very small areas*, within which the absorbing substances are evenly distributed. *Caspersson* has developed a technique for measuring accurately transmissions in areas as small as 0.1 square μ. This necessitates the use of a high-aperture objective fulfilling *Abbe*'s sine condition, and the light-measuring instrument must be very sensitive and accurate. A large aperture is necessary

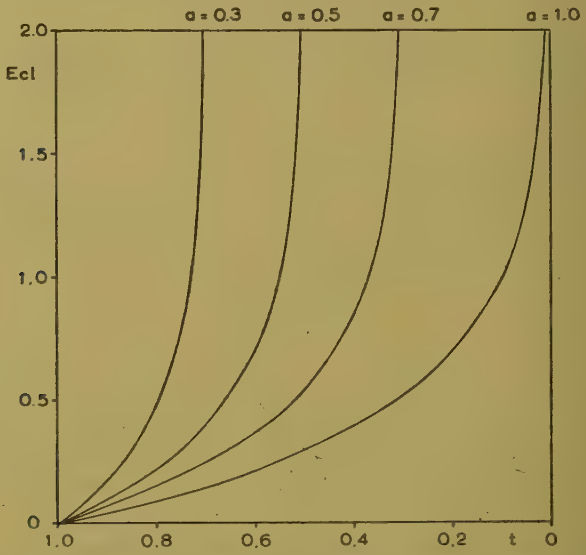
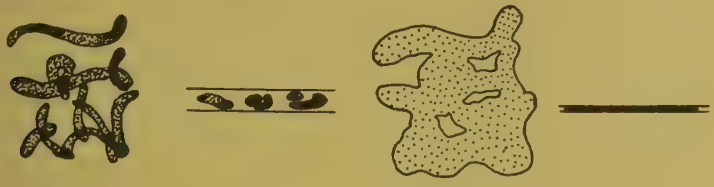


Fig. 23. Ecl as a function of *t* when the absorbing material covers a fraction *a* of the total area.

not only for collecting most of the scattered light but also to obtain a correct intensity distribution in the image plane. The object must also be illuminated with incoherent light, which is the case when Köhler's illumination or diffuse light is used.

If it is wanted to know the average concentration of some absorbing substance in a nonhomogeneous specimen, several measurements are to be carried out in small areas distributed *at random* over the entire area of the specimen. This method is a combination of the point-sampling method (page 67) and of absorption measurements. The average concentration can be calculated from the average extinction, which was seen above not to be equal to the negative logarithm of the average transmission.



a) b)

Fig. 24, illustrating the effect of crushing on chromosomes. The density decreases, the absorbing area increases, and differences in the magnitude of absorption are greatly diminished. a) before crushing; b) after crushing.

As many absorbing constituents such as chromosomes, nucleoli, etc. are very small, difficulties are met in trying to perform the measurements in a field small enough to assure homogeneity. *Davies, Wilkins* and *Boddy* (1954) have suggested the use of compression in such instances. The method is illustrated in Fig. 24. Compression simultaneously increases the homogeneous area and guarantees an equal specimen thickness over this area. In practice the compression is done by means of a specially designed condenser with a spherical top lens. The slide is placed on the microscope stage the plastic cover slip downwards; by elevating the condenser the space between the slide and the cover slip can be diminished at will.

While the last-described method is particularly useful in studying small tissue constituents, the reverse, *increasing the thickness* of the specimen is useful when absorption measurements are carried out on large areas of sections. Increasing the thickness of a section is analogous with increasing the cuvette thickness in our earlier example of blood photometry. The absorption by the erythrocyte mixture will be even over the whole cuvette, if the absorbing layer is thick enough. In the same way, the sections can in many instances be prepared sufficiently thick to abolish complications due to nonhomogeneity. Thick sections have the advantage that errors in the estimation of the average section thickness are smaller. Furthermore, individual parts in the same section deviate less from this mean than in a thin section. Unless refractive index differences within the section are very small and can therefore be neglected, due caution is necessary not to overlook errors due to light scatter. Absorption measurements on thick sections are conveniently done by inserting the sections directly on a diffusing glass, as described earlier, and using a low-power objective with a small numeric aperture. In this way, the additional advantage is reached that the probability of obtaining a representative sample is much increased in comparison to that obtainable by making several extinction estimations on randomly selected points of a thin section.

The method may sometimes prove difficult because of the high extinction accompanying the thick absorbing layer. In the visible range this difficulty can mostly be overcome either by making the measurements at wavelengths where the absorption of the substance concerned is relatively low or by using stains with low molar extinction. In ultraviolet light extinctions of unstained specimens are generally low. High extinctions furthermore need not affect the accuracy of the measurement, although the contrary might be expected knowing that, with a constant photometric error, the error in the estimated density tends to become excessively high at high and low transmissions. This kind of instrumental error can be avoided by the method of density differences as long as the sensitivity of the light-measuring instrument is sufficient.

Method of Density Differences.

The value of transmission can be obtained by measuring first the intensity of the light passing through the optical system without the specimen and then the intensity of the light which is transmitted the specimen in position. It is clear that in this case a stable light source must be used. Alternatively, compensating circuits (see chapter X) may be used in which the intensities of two light beams are simultaneously compared, one beam—called the reference beam—passing the optical system only and the other beam—called the sample beam—passing the same optical system and the specimen. If the reference beam is allowed to pass the slide, the cover slip and a non-absorbing part of the specimen, the transmission is smaller than if the beam passes the microscope without the slide. This difference is obviously due to loss of light in the slide, in the specimen and in the cover slip. As this loss obeys Lambert's law, the following equation applies:

$$I' = I_0 10^{-a} 10^{-b} 10^{-d} = I_0 10^{-(a+b+d)}, \tag{50}$$

where I_0 is the intensity of the light measured without the slide, I' the intensity measured through a non-absorbing part of the specimen and a, b and d constants depending on the properties of the slide, the specimen and the cover slip, respectively. Let E be the extinction coefficient of the substance whose concentration c in the specimen with a thickness of l is wanted to measure. Presuming that Beer's law is valid, the intensity of light in the sample beam is then

$$I = I_0 10^{-(a+b+d)} 10^{-Ecl} = I_0 10^{-(a+b+d)-Ecl}, \text{ and}$$

$$\frac{I}{I'} = \frac{I_0 10^{-(a+b+d)-Ecl}}{I_0 10^{-(a+b+d)}} = 10^{-(a+b+d)-Ecl+a+b+d} = 10^{-Ecl}, \text{ or}$$

$$-\log \frac{I}{I'} = Ecl. \tag{51}$$

Thus, by using as a reference beam a beam passing through such parts of the specimen causing a loss of light not related to the substance to be studied, the concentration of this substance can be calculated from the simple equation (51) without regard to the source or magnitude of the non-specific loss of light. The condition of course is that the non-specific loss is equal in the non-absorbing part and in the absorbing part of the specimen.

This method will be found useful when great losses of light occur because of light scatter. I' is first measured in the desired part of an unstained section. The section is then stained or treated according to the histochemical procedure and I is measured in the same part of the stained section. Although only a fraction of the light transmitted by the concerning part of the specimen had entered the objective, no error due to light refraction or reflection will interfere with the result (unless the treatment has resulted in alterations in the light-refracting properties of the specimen). Accurate location of the specimen is naturally necessary and the measurements must be carried out under otherwise strictly identical conditions, the section mounted in the same way both times. At low magnifications readings of the vernier scale of the slide holder can be used for location. At high magnifications the site of a characteristic point of the specimen in the visual field must be noted, e.g., with the aid of a hair-cross network fixed in the ocular. For accurate location of the specimen, see also *Caspersson* (1950).

In a thick, heavily absorbing section, the high density may diminish the accuracy of the measurement. This can be prevented by letting the reference beam pass through a cuvette containing some absorbing fluid, e.g. a solution of the dye stuff with which the section has been stained. Let the section thickness be l, the concentration of the coloured substance in the section c, and the extinction coefficient of the substance E. Let further the thickness of the cuvette be l', the concentration of the solution in the cuvette c', and the extinction coefficient of the substance in the cuvette E'. Then the density measured is

$$d = -\log \frac{I}{I'} = Ecl - E'c'l', \tag{51a}$$

or, denoting $El = a$ and $E'c'l' = b$,

$$d = ac - b\,.$$

As a and b are constants, the density is still linearly proportional to the concentration, presuming of course that Beer's law is valid.

CHAPTER XI

Equipment for Absorption Measurements

Alternative Arrangements.

A complete spectrophotometer for microscopical work contains the following parts:

1. light source,
2. monochromator
3. microscope, and
4. photometer.

Figs. 25-29 show some alternative arrangements. In Fig. 25 the light passes through a monochromator into the microscope, and a part of the magnified image of the specimen is projected on a light-sensitive phototube. In Fig. 26 the specimen is illuminated with heterochromatic light and the magnified image is focused on the entering slit of a spectrograph (*Barer*, *Holiday*, and *Jope*, 1950). Fig. 27 illustrates a measuring system in which a narrow hole on the exit slit of the

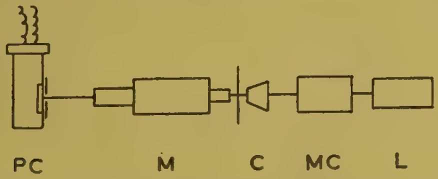


Fig. 25. A simple arrangement for section photometry. L, lamp; MC, monochromator; C, condenser; M, microscope; PC, photocell.

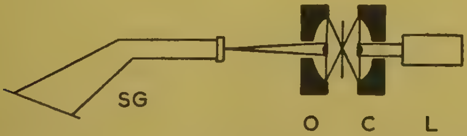


Fig. 26. Spectrographic apparatus in which the specimen is illuminated with heterochromatic light. L, lamp; C, mirror condenser; O, mirror objective; SG, spectrograph.

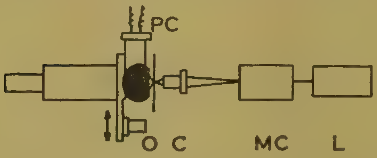


Fig. 27. A photometric device in which the light transmitted by the specimen is directly measured with a phototube. The microscope is used only for focusing the condenser and for selecting the field. L, lamp; MC, monochromator; C, high-power objective serving as a condenser; O, objective; PC, photomultiplier tube.

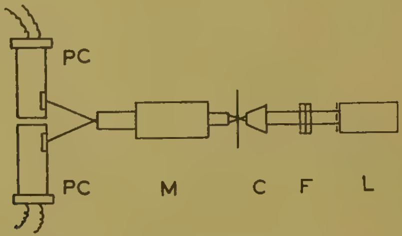


Fig. 28. A two-beam photometer. Both the sample beam and the reference beam are passing through the specimen. L, lamp; F, a filter combination; C, condenser; M, microscope; PC, photocell.

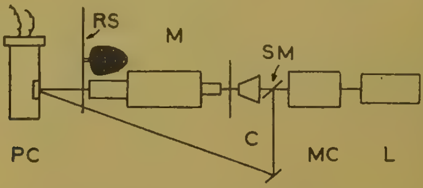


Fig. 29. A single-tube compensating microphotometer. The light beam coming from the monochromator, MC, is split into two beams by means of a semi-reflecting mirror, SM. One of these beams passes through the condenser, C, the specimen and the microscope, M. Both beams go through a rotating sector, RS, which gives different frequencies to them. Although both beams stimulate the photomultiplier tube, PC, different frequencies allow separation and compensation by electronic means.

monochromator is focused by the condenser on the specimen and the microscope is used only for focusing, while photometry is performed by replacing the objective with a phototube enclosed in a light-diffusing sphere. By this means scattered light can be collected in a wide angle. Fig. 28 and Fig. 29 show arrangements in which compensation is provided for fluctuations in the intensity of light. In Fig. 28 both the sample beam and the reference beam pass through the slide and the intensities of both beams are matched with two phototubes in a compensatory circuit. A combination of filters is used as a simple monochromator. In Fig. 29 the reference beam does not go through the optics at all, and both beams enter the same phototube. A rotating sector is used to provide an intermittent light. The photocurrents induced by the two beams can be electronically separated and compensated.

Several other possibilities could be presented. Which combination is most useful in each instance depends on a great number of factors, frequently not least on the limit of financial resources.

Light Sources.

The spectral range to be used is a decisive factor in the choice of the light source. The spectral intensity distribution of the lamp must be in a sensible relation to the corresponding sensitivity distribution of the light-measuring device. A further condition, set by microscopic work, is that the intensity per unit of illuminating area must be high.

In the visual range, incandescent lamps are the source of choice for the majority of cases. Bulbs manufactured primarily for other purposes are cheap and many of them are fully satisfactory for microscopic work. The filaments should be so arranged that a small area will be nearly evenly illuminated. Such bulbs are commercially available amongst others for the use in projectors. The useful wavelength range of such lamps extends from about 4500 Å to near infrared. The wavelength of the maximum intensity can be lowered to a certain degree by increasing the voltage applied across the filament. Exceeding the voltage prescribed by the manufacturer results in premature deterioration of the lamp but temporarily overvoltage can be used to increase the radiation intensity at lower wavelengths.

Mains voltage shows everywhere too large fluctuations to allow the use of this convenient source for feeding the lamp, unless compensation is provided for the light-intensity fluctuations. Even so, changes in the colour temperature of the source may alter the spectral characteristics of the light to a deleterious degree. Rough stabilization can be achieved with a constant-current transformer but the remaining fluctuations still necessitate balanced methods of measurements. Electronic stabilizers give a sufficiently constant lamp current.

In most instances a storage battery of high capacity is most convenient as a stable source of electricity. Care must only be taken not to work with a nearly exhausted battery, because even small changes in voltage radically affect the light intensity, proportional to nearly the fourth power of voltage.

For work in the ultraviolet other sources of light must be used. For many purposes mercury-vapour lamps of the street-lighting type are satisfactory. When higher intensities are required, high-pressure mercury-vapour lamps or, below 2,500 Å, rotating spark gaps or metal arcs may prove useful. The stability of these sources is generally low wherefore efficient compensating methods of measurement are obligatory. Recent models of hot-cathode hydrogen-discharge tubes are characterized by continuous spectrum in the ultraviolet, steady light intensity and low cost. Though the intensity is low, they may be used with advantage in some applications, e.g. in a spectrographic system similar to that illustrated in Fig. 26.

Monochromators.

Monochromatic light can be obtained with prism monochromators, diffraction-grating monochromators, or with filter combinations. When discontinuous light such as that obtained from the mercury-vapour lamp is used, a high spectral purity can be achieved by relatively simple line-isolation methods. The desired degree of spectral purity is a complex function of the wavelength-intensity distribution of the light obtained from the source, of the absorption spectrum of the substance to be estimated, and of the spectral-sensitivity characteristics of the measuring device. Work in the ultraviolet necessitates good spectral isolation and possibilities for performing measurements at closely neighbouring wavelengths. The monochromator or spectrograph must accordingly be of sufficently high quality. This means generally that the monochromator is better purchased from an optical company. Satisfactory ultraviolet monochromators can nevertheless be self-constructed for many purposes by using water in quartz cuvettes as the dispersing medium (see *Strong*, 1949).

In the visible range sufficiently monochromatic wavebands can mostly be isolated by means of filters or filter combinations. This is particularly true when density measurements are carried out on a stained preparation completely colourless except for the stained areas. Narrow wavebands are of course necessary also in the visible region when it is desired, e.g., to investigate changes in the absorption spectra of stains in the tissues.

Coloured liquids in plane-parallel cuvettes make good filters easily prepared in a histological laboratory. In using such filters it is good to bear in mind that the absorption characteristics of many synthetic dyes tend to alter under irradiation. The same applies to some gelatin filters, which are otherwise an economical and efficient solution to the filter problem. Glass filters show the highest stability and a set of some 10 filters properly selected is enough for many purposes. Approximate spectral characteristics of filters are usually given in the manufacturer's catalogues, which also frequently contain interesting information to those who want to use self-prepared liquid filters. It must be remembered that for accurate work the absorption spectra of each individual filter must be measured with a good spectrophotometer.

For isolation of narrow wavebands interference filters are superior to colour filters in giving a high transmission—round 0.3—at a narrow waveband only. Although the transmission is only a fraction of this value outside a narrow range, a transmission of about 0.01 at wavelengths some 500 Å from the wavelength of maximum transmission may cause gross errors. This happens if e.g. the intensity of the light source, the sensitivity of the measuring instrument, and the transmission through the substance to be measured are high at a wavelength where the filter should not transmit.

While filters transmitting only a narrow waveband are needed when the end-point of the measurement is photographic or photoelectric, the same does not apply when the light intensities are visually measured. The same subjective colour sensation can be caused by two light spots whose spectral composition is altogether different. Thus, highly monochromatic light may apparently be of exactly the same colour as heterogeneous light. Therefore, when light intensities are visually matched, spectral purity is not of decisive importance and wide-band filters may be used. On the other hand, visual inspection is a poor guide in the selection of narrow-range light filters.

Microscopes.

When very small areas of cells are analyzed with spectrophotometric methods, a sharp image of the object is necessary to be able to isolate parts of this image for light intensity measurements. Unless the light-intensity distribution in the image is equal to that immediately behind the object, false results are obtained. Ideally, the optics should give a sharp image irrespective of the wavelength of the light. Lens errors such as aberrations and coma should be negligible and the aperture should still be high (cf. page 92). This condition can only be fulfilled by optics operating solely with reflecting mirrors which give an equally sharp image at any wavelength. Several reflection microscopes intended for work in both ultraviolet and visible light are commercially available. *Barer*'s (1950 b) article seems, however, to indicate that none of these microscopes approaches the completeness required for spectrophotometric work. The reflection microscope designed by *Burch* (1947), on the other hand, seems to satisfy the conditions briefly outlined above and it has been used for studies both in the ultraviolet and the infra-red region of the spectrum. This microscope is at the moment produced on a very small scale and it is accordingly fairly expensive. Because of the unique characteristics of this microscope it can only be hoped that production on a larger scale will make it accessible to wider circles. In this connection it is adequate to note that the arrangement shown in Fig. 26, page 101, presumes that the microscope used gives a sharp image over all the spectral range to be analysed.

None other but reflection microscopes are capable of working satisfactorily over the whole spectral range. Ordinary, so-called achromatic objectives are actually monochromatic and give a fully satisfactory image at one wavelength only. Therefore, the distribution of light in the image plane at wavelengths only a few millimicrons away from the optimum is no more equivalent to that in the object plane, in spite of correct focusing. This is likely to cause false results, particularly in the ultraviolet region, where the magnitude of error is difficult to estimate (see *Barer et al.*, 1950). For the maximum accuracy, therefore, separate achromatic objectives are theoretically necessary for each wavelength. According to *Caspersson* (1950), nevertheless, two achromatic quartz objectives are satisfactory for measurements from 2,020 to 3,300 Å.

While ordinary achromatic objectives are suitable for a restricted spectral band only, *Boutry* and *Billard* (1947) have designed so-called polychromatic objectives, which are correct over a wide range of wavelengths. Focusing must be done at each wavelength separately as a sharp image is not obtained simultaneously at different wavelengths.

The best lens-combination objectives in the visual range are generally called apochromatic. They are corrected at three different wavelengths and work reasonably well at the intermediate wavelengths also. Qualities of apochromates of different make are very variable, however, and it may be demonstrated that with some apochromates considerable refocusing must be done when the wavelength is changed.

When Köhler's illumination or, in particular, when diffused light is used, the optical properties of the condenser need not be of as high standard as those of the objective. However, inaccuracies in the optics below the object may cause considerable errors, particularly if the illuminated field is restricted to diminish the stray light caused by reflections in the optical system. Such errors easily escape notice. To be on the safe side the condenser may therefore be replaced with an objective similar to that used for producing a magnified image of the object (*Naora*, 1951). A faultless condenser is indeed obligatory when the measurement of light is carried out directly above the object (Fig. 27, page 102), instead of doing it in a restricted part of the image formed by the microscope.

Photometry in Visible Light.

Although the most revolutionary results have been obtained in the ultraviolet region of the spectrum (*Caspersson*, 1950), several

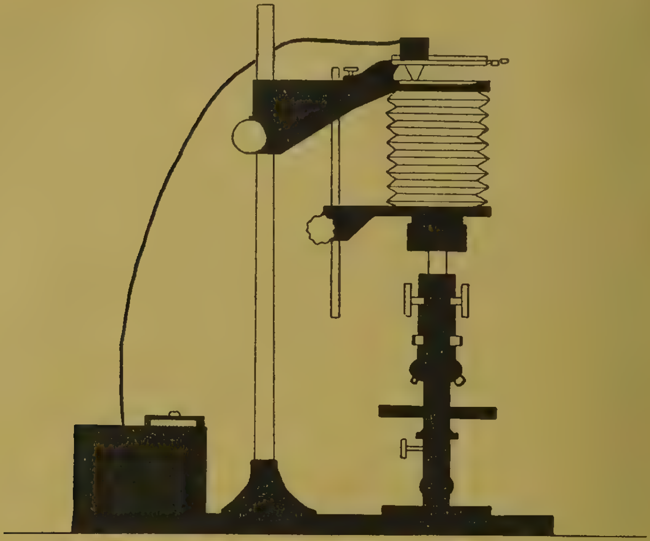


Fig. 30. A photometer described by *Dempsey et al.* (1947). A phototube can be moved on the ground glass of the photographic system.

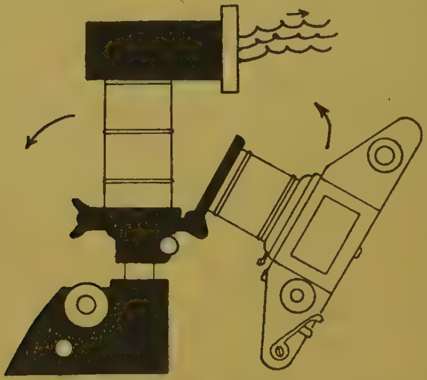


Fig. 31. Another arrangement in which the specimen can first be focused and photographed with a reflex camera and then measured for absorption with a phototube.

studies have proved that photometry of stained or histochemically treated sections in visible light is of great interest. The considerations in this volume have been given mainly with this kind of measurements in mind, because they are possible in some form in practically every laboratory. Detailed discussion of spectrophotometric aspects connected with specific staining methods or histochemical reactions fall however outside the scope of the present volume. Additional information can be obtained in the papers already referred to and in articles by *Stowell* (1942, 1945 a, b, 1946), *Stowell* and *Albers* (1943), *Pollister* and *Swift* (1950), *Pollister, Himes* and *Ornstein* (1951) (general and nucleic acids); *Deane, Nesbett* and *Hastings* (1946) (glycogen); *Dempsey, Bunting, Singer,* and *Wislocki* (1947) (mucopolysaccharides); *Metcalf* (1951) (haemoglobin); and *Abolinš* (1952) (phosphatase).

Figs. 30 and 31 show two practical arrangements for visual-range photometry according to the same principle as in Fig. 25, page 101. In both of these systems, the specimen measured can be conveniently photographed.

CHAPTER X

Measurement of the Intensity of Light

Introduction.

The intensity of light can be measured with three different methods: 1. visually, 2. photographically, or 3. photoelectrically. Visual measurements are ordinarily restricted to the region of the visible spectrum. With the aid of fluorescing substances or the recent image-converter units it is nevertheless possible to extend visual estimations to ultraviolet and infra-red parts of the spectrum. Photographic and photoelectric methods can be applied to regions from far ultraviolet to near infra-red and to far infra-red when image-converters are used. Thermocouples, which will not be discussed further, are used for measurements in the infra-red region.

Before going into details, some general statements about the accuracy of the measurements may be useful. A quantitative technique which is both sensitive and accurate is naturally useful for all purposes. However, most techniques tend to lose accuracy when the sensitivity is much increased and vice versa. Frequently refinements of the technique are possible only at high cost and tend to make the actual measurements complicated and cumbersome. Therefore, it is wise to form an idea about the accuracy needed for each particular problem.

For example, it may be that the standard error in estimating the section thickness is about 5 per cent. Reduction of the error in absorption measurements to zero would still keep the total error at 5 per cent. On the other hand, if the error of the absorption measurements also amounts to 5 per cent, the total error will not be 10 but $\sqrt{5^2+5^2} = 7.07$ per cent (see formula 11, page 21), which is not much more than 5 per cent. This shows that relatively little is gained in overall accuracy by reducing one kind of error only. Extinctions in tissue sections may show very large variations not related to differences in the concentrations. In such instances simple assessments with primitive equipment may give equally useful data as a superfine microspectrophotometer.

Physiological Basis of Visual Photometry.

Although the eye is very sensitive in creating sensations when illuminated with a low light intensity, only rough estimates of *absolute* intensities of light can be done visually. Differences in the intensities of two sources of light can, on the contrary, be noted with high accuracy, if the sources are simultaneously examined. The difference threshold of intensity is dependent on the absolute intensity of light. It is generally agreed that under optimum conditions the least perceptible difference between the intensities of two surfaces is less than 1 per cent of the absolute intensity of light. This applies to medium light intensities only. Both in very strong and in poor light the difference threshold is higher in relation to the absolute intensity, i.e., only big differences are appreciated. Within a large range of illumination the difference threshold keeps nevertheless reasonably proportional to the absolute intensity of light (*Weber*'s law). This implies that the percentage error is within that range largely independent of variations in the intensity of light.

Holiday (1950) has drawn attention to the fact that subjective assessment of the light intensity on a small area depends on the light intensity on closely neighbouring areas. Thus, in a step wedge an area with even density appears subjectively lighter near the neighbouring step with higher density and conversely darker near the other neighbouring step with a lower density. Using this contrast effect, small differences in the density of a photographic plate can be detected, undetectable by photoelectric densitometry. This phenomenon is important in subjective microscopic photometry and has to be taken into consideration in assessing sections with irregular distribution of the absorbing materials.

Simple Assessment of Absorption.

In ordinary histological and histochemical research it is customary to express differences in the light absorption with terms such as "weakly stained", "dark brown", "strongly positive", etc. Recently, there has been increasing tendency to quantitate these differences subjectively, and the results have been given accordingly to an arbitrary scale. The scale may range from 0 to 5 or from + to +++.

This quasi-quantitative technique is simple and it does not need any accessory equipment. The accuracy is of course low. Furthermore, the value of the information thus obtained is essentially dependent on the skill of the observer. The significance of the results may also be rather difficult to assess, especially to another worker reading the report concerned. Anyhow, if certain precautions are taken into consideration, this method can be used to extract valuable information from tissue sections.

Before the assessments are started, one has to make clear the significance of the symbols used to indicate the degree of absorption. If this is not done, an area may be recorded to fall into, say, class 3 in the beginning while a similar area is in the end put into class 5. Errors thus created can be essentially diminished by referring the classes to some abstraction serving as a primitive standard for the assessment. As an example, the scale used by *Macdonald* (1951) is reproduced here.

0	No reaction.
1	Yellow colour.
2	Brown colour.
3	Black colour with the nucleus readily distinguishable.
4	Black colour obscuring the nucleus.

The method was used to assess the intensity of histochemically demonstrable acid phosphatase in the cytoplasm of nerve cells. Statistical analysis indicated a reasonable reproducibility of the results.

The accuracy can probably in many cases be further increased by the use of physical instead of abstract standards. Slide or slides with previously marked areas or cells falling into different classes can be used as fixed standards throughout the assessment. Ink marks and readings of the vernier scales of the slide holder can be used to locate the reference sites.

Examination of a slide should be done without knowledge of its source. To avoid errors due to the possible, continuous change in the subjective criteria it is preferable to mix all slides, instead of first assessing the control slides and then the experimental ones. If possible, several estimations should be done on each slide, and each slide should be examined at least twice. If the purpose is to assess the absorption of some tissue component distributed over the whole section, the sites of assessment should be selected at random. During the evaluation of the whole material the same optics and illumination conditions have to be used.

There is hardly any other field where statistical treatment of the results would be more useful than in subjective assessment of sections. Especially if many classes are used, it is quite possible that even large differences in average "absorption" are not statistically significant. It is permissible to calculate an average value of "absorption" in each section or group of sections. One has to bear in mind, however, that such numerical values have no absolute significance, although they allow the use of statistical methods. Before applying the subjective assessment to an extensive material, it is advantageous to make a series of double estimations in a small number of sections to see whether the differences in absorption are in comparison to the accuracy of assessment big enough to justify the use of 5 instead of 3 classes, or any use of this method at all.

Coujard slides.

If the intensity of blackening is assessed according to an arbitrary scale, the estimates do not bear any simple relation to the magnitude of extinction. Moreover, in many instances all-or-none reactions play an important role in histochemical reactions responsible for the staining of sections so that relations between the extinction and the amount of reactive material present are far from linear. As *Gomori* (1950) has shown, it is nevertheless possible to make approximate estimations in terms of concentration or chemical activity by using dilution standards on slides treated simultaneously with the sections according to the histochemical procedure.

On slides treated with egg-white glycerol, and dried, marks are made using dilutions of the substance to be demonstrated. Serial dilutions are made with e.g. 1 per cent gelatin or gum acacia, and a clean steel pen is used to make the marks. The thickness of such lines can be calculated from their width using an empirically constructed curve. The number of the dilution fraction can be conveniently used as a mark for each "ink". Control slides thus prepared are thereafter treated identically with those carrying the sections to be assessed. After the histochemical procedure the intensity of darkening in the tissue is compared with that of the dilution marks. To avoid errors due to uneven distribution of absorbing materials (see page 94) each component to be assessed must be homogeneous. The dilution mark having the same depth of "staining" is noted. If the thickness of the tissue component concerned is equal to that of the mark, the concentration of reactive materials is regarded equal also. In case the thicknesses are different, the chemical activity of the detail was supposed by *Gomori* to be equal to that of the dilution mark showing the same blackening multiplied by the ratio (thickness of the tissue layer/thickness of the mark). This method was said to be capable of discriminating acid phosphatase activity with an accuracy of about ± 50 per cent.

Theoretically the method is quite sound as long as the thickness of the dilution mark is equal to that of the tissue constituent to be compared with it. If the ink mark is thicker, correct results can only be obtained, if Beer's law (page 88) is valid. Suppose a tissue component showing an equal darkening with that of an equally thick dilution mark 3 indicating the activity level of a dilution $1 : 10^3$. If the dilution mark were 5 times as thick as the section, a correct result is obtained only when an equal extinction is seen in a mark diluted $1 : 10^3$ and a mark 5 times as thick diluted $1 : 5 \times 10^3$, i.e. when the extinction in the mark is proportional to the concentration of the reacting substance. This is almost certainly not the case for histochemically demonstrated phosphatases and for many other histochemical reactions.

There are further sources of error, which may distort the results unless the thickness of the calibration mark is kept equal to that of the section. Although Beer's law could be demonstrated to be valid under the prevailing conditions, care must be taken, if the thickness of the calibration mark is different from that of the tissue part involved. If the reacting substances are to a certain degree soluble in the reagents used, the percentage amount left in a thin layer is much smaller than that in a thick layer. In many histochemical enzyme methods a certain minimum thickness of the section is indeed necessary to get any reaction at all. On the other hand, the time required for the reagent to diffuse in all parts of a thick layer is longer than that in a thin layer. Thus the colour-producing reaction may be completed in a thin section before the reaction has started in the inner parts of a thick calibration mark.

Sometimes it may be difficult to obtain equal thicknesses for the section and the calibration mark. Although it is not justified in such case to express the activity of the tissue in terms of dilution—unless such relation is directly proved—the technique will still be valuable because the dilution marks serve as standards, whose darkening is dependent on similar chemical processes as that of the section.

Gomori (l.c.) used separate slides for the control marks. To abridge the time lag between the inspection of the marks and the sections it might be of advantage to draw the marks on the same slide with the sections, preferably near them. Reduction in the inspection interval increases the accuracy of comparison. Alternatively and still more conveniently, the standard slide could be kept constantly under another microscope with identical optics and illumination. In this case, a comparison ocular could be used to examine the two slides simultaneously.

Visual Photometry.

While the afore-mentioned methods are necessarily limited in accuracy because the visual comparison of the sample and standard is alternating, simultaneous matching of two light sources makes full use of the high discriminating sensitivity of the eye. The principle is the same in all modifications of visual photometry. The light intensities of two surfaces are compared simultaneously and their mutual intensities are adjusted until no difference in the intensity can be observed. Reading of the adjusting device can be calibrated in terms of extinction or of transmission. The light intensity can be varied by the use of

a) polarized light,
b) rotating sectors,
c) absorption wedges,
d) absorbing solutions,
e) Lambert's inverse square law, or
f) varied apertures.

a) *Polarized light.* In the visual photometry of solutions the use of polarized light is by far the most popular method. Fig. 32 illustrates two methods by which polarized light can be used to equalize the light intensity of the light beam passing the absorbing layer with that of the reference beam. In the first one, the intensity of the reference beam only is altered.

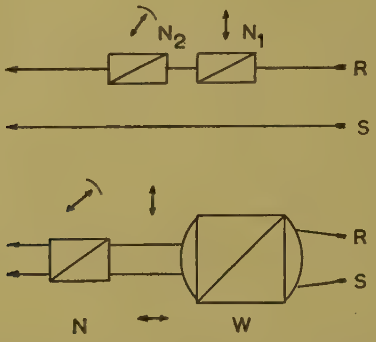


Fig. 32. Two arrangements for altering the mutual intensities of the sample beam, S, and the reference beam, R. In the upper arrangement, the nicol N_2 is rotated until the two beams match. In the lower system the beams polarized by the Wollaston prism, W, at directions perpendicular to each other are matched by rotating the nicol N.

Instead of nicols, polaroid filters can also be used. In most instances they are adequate enough, and the expenses are much smaller. It is wise to calibrate the transmittance at various angles with the aid of a spectrophotometer at different wavelengths. If such device is not available, a simple method is to make use of the inverse square law (see below). In many makes of polaroid sheets the crossed filters do not absorb red and infra-red radiation. This does no harm, of course, when measurements are done at lower wavelengths and monochromators are used. In simple colorimetric systems, the error can be corrected by inserting a filter.

The other arrangement makes use of the Wollaston prism, which plane polarizes the light into two beams, the polarization planes of which are perpendicular to each other. The light intensities in the two beams can be matched by rotating a nicol in front of the prism. The same result can naturally be achieved by inserting two polaroid sheets with the polarization planes perpendicular to each other in front of the light source instead of the Wollaston prism and by replacing the nicol with a polaroid filter.

b) *Rotating sectors.* If the passage of light is periodically interrupted by means of a rotating sector and the frequency of the produced flicker is high enough, the light intensity transmitted *(I)* seems subjectively even. The sensation is equal to that caused by a continuous light having the intensity given by the following equation, called Talbot-Plateau law:

$$I = \frac{a}{a + b} I_0 , \tag{52}$$

where a = free angle of the sector, b = screened angle of the sector. Any high-speed motor is suitable for the rotation. If a-c operated light sources are used, the flicker frequency chosen must be different from that in the mains voltage to avoid interference phenomena. Otherwise variations in the frequency do not influence the resultant intensity of the sensation once the critical fusion frequency is exceeded. Frequency higher than 40 cycles per second is usually sufficient to cause fusion but higher frequencies can easily be obtained by cutting several sectors in the rotating circle.

c) *Absorption filters and wedges.* In the same way as by the use of two nicols the light intensity can be controlled with the aid of neutral filters absorbing light throughout the whole visible spectrum. Such neutral filters can be made by exposing photographic plates for varying periods or to varying intensities of light. The emulsion side of the exposed, developed, and fixed plate is then covered with a cover slip using canada balsam. A series of such filters with varying density can be attached to a circular disc and the approximate absorption of the sample may be measured by changing filters in front of the reference beam until this and the beam passing the sample are approximately equal in intensity. Instead of a series of filters a step wedge can be prepared in an identical way on a single photographic plate.

Unless a large number of filters, or of steps in a step wedge, is used, the absorption can only be roughly measured. The accuracy can be increased by the use of continuous wedges, a small part of which is in front of the narrow reference beam at a time. Such a wedge is conveniently prepared by raising a photographic plate at a logarithmically increasing rate perpendicularly to a narrow slit illuminated with constant intensity. All filters and wedges made from photographic plates must be repeatedly calibrated, as their absorption tends to change.

Neutral wedges may also be prepared by vacuum-coating thin layers of metal on glass. Coloured glasses with a suitable absorption spectrum are equally useful and more stable than any of the neutral filters. Commercially available comparator disks can sometimes be equally well used for microscopical absorption measurements.

As a whole, the estimation of the absorption with the aid of filters and wedges is quasi-quantitative rather than quantitative. This is not necessarily a serious drawback, as many other factors such as deviations from Beer's law, light diffraction and uneven distribution of absorbing materials in the section may non-specifically influence the absorption to such a degree that very accurate methods for measuring the light intensity become superfluous.

d) *Absorbing solutions.* By letting the reference beam pass through a solution having the same colour as that of the substance in the section whose absorption is to be measured, the light intensities in the sample and reference beams can be matched. This is done most conveniently when a cuvette of adjustable depth is available. If such a cuvette is, e.g., filled with a solution of the same stain which is responsible for the absorption in the section, the amount of absorbing material in the section is directly given by the product of the depth of the cuvette und the concentration of the solution. The condition of course is that Beer's law is obeyed and that the distribution of the staining is even in the measured part of tissue. If Beer's law is known to be not valid, reliable results can only be obtained, when the concentration of the coloured substance is the same both in the reference cuvette and in the section. In practice this means that a very thin cuvette should be used because the concentrations of absorbing materials are usually high in tissue sections. High extinctions prevent the use of two cuvettes, one of which is placed to diminish the intensity of the sample beam and another, a little thicker, to control the reference beam. Photometry is much easier, if an empirically constructed calibration curve can be used for correcting deviations from Beer's law.

Instead of cuvettes with variable depth wedge cuvettes can be used in the same way as a continuous wedge.

e) *Inverse square law.* One arrangement is illustrated in Fig. 33. The image of the microscopical specimen is projected on one half of an opal glass, divided into two fields by means of a separating wall. The size of the field is adjusted with an iris diaphragm until the image of the specimen is homogeneous. The other half of the field is illuminated by a small lamp, whose distance from the opal glass can be altered. This lamp is moved till the two parts of the examined field show an equal intensity. Using Lambert's inverse square law the light intensity can be easily calculated. Appropriate filters must be used to match the colours in the two fields before actual measurement.

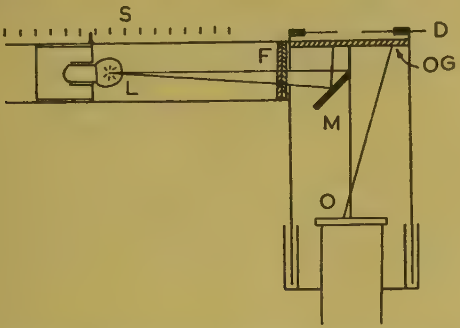


Fig. 33. A simple device for photometry using the inverse square law. D, iris diaphragm; F, filter combination; L, lamp; M, mirror; O, ocular; OG, opal glass; S, scale for reading the distance of the lamp from the opal glass. The image of the specimen is focused on one half of the opal glass, separated from the lamp system with a vertical wall. The lamp is then moved until the intensities on both sides of the wall match.

f) *Varied apertures.* If a variable aperture is placed between two opal glasses, the illumination can be conveniently regulated. The loss of light must be empirically determined because opal and ground glasses may pass variable amounts of direct light. The use of variable apertures without opal or ground glasses is not possible without changing the optimal microscope illumination. When this method is used, great losses in the intensity of light occur.

Photographic Methods.

The blackening of the photographic plate is dependent on the light energy reaching the sensitive emulsion. Fig. 34 shows a typical curve, in which the density of the plate is plotted against the exposure.

Certain blackening can be seen in a developed plate which has not been subjected to light at all before the development. This basic blackening is called *fog*, and it can be increased by prolonged development. Very small amounts of light do not increase the density above this fog level. The minimum exposure causing a further darkening of the plate is called *inertia.* With certain values of exposure the density is directly proportional to the logarithm of the exposure. The slope varies in different kinds of plates and it is expressed in terms of $\gamma = \tan \alpha$.

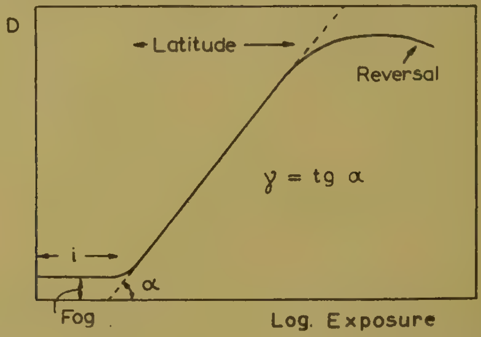


Fig. 34. A typical γ-curve of a photographic emulsion.

The exposure is a function of the intensity of light *(I)* and the length of time during which the plate has been subjected to the light. If the exposure time *(t)* is kept constant, the *density* (see page 88) of the plate is proportional to the intensity of light.

The reciprocity law of Bunsen and Roscoe states that the effective exposure is equal to the product of the light intensity and the time of exposure, or $I\,t$. Indeed, if the exposure time is only little varied, the density keeps within the limits of the accuracy of the density measurement when $I\,t$ is constant. *Schwarzschild* (1899) observed, however, that with low intensities of light it is necessary to expose the plate for a longer time than that given by the reciprocity law. He suggested that $I\,t$ should be replaced by $I\,t^p$, where p is 0.8. In this case the density could be calculated from the following formula:

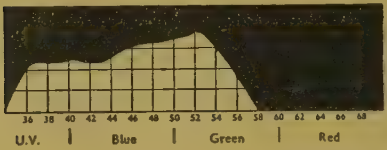
$$D = \gamma \log I\,t^p - i, \tag{53}$$

where D = density and i = inertia. Since then it has been shown that with various values of intensity, p may vary several tenths. Furthermore, at a certain intensity, which varies in different plates, the same density is achieved with a lower value of log $I\,t$ than at higher or lower intensities.

Reciprocity law is fulfilled at temperatures less than —186° C (*Mees*, 1942). For most emulsions at room temperature there is an intensity range within which log $I\,t$ keeps fairly constant.

Failure to follow the reciprocity law makes it understandable that intermittent light such as that produced by means of a rotating sector may produce variable densities although the total light radiation is kept constant. If the frequency of the flicker is high enough (over 50 revs/sec.), the resulting density is nevertheless proportional to the ratio (free angle of the sector total angle) with a constant intensity of light and constant exposure time.

A large variety of plates and films is now available. Their characteristics with regard to the sensitivity (inertia) at different wavelengths, resolving power (graininess), and contrast (slope of the exposure-density curve) are reported by the manufacturer. Fig. 35 shows data of a very slow, ultraviolet- and blue-sensitive, high-resolution and -contrast plate. The time-temperature curve gives the range of developing times and temperatures producing the same γ. Fig. 36 illustrates characteristics of a panchromatic high-speed sheet film with a low γ and a medium graininess. It must be noted that this kind of information can only be regarded as an approximate guide to the characteristics under specified conditions of measurement. Thus, e.g., the spectral sensitivity is usually indicated by wedge spectrograms obtained using light from a tungsten lamp and glass optics. The actual maximum sensitivities of the emulsions are in the ultraviolet, to which glass is not transparent. Even so, the shape of the spectral sensitivity is affected, if another kind of light source is



Sensitivity Curve of Tungsten Light (2848° *K.*)

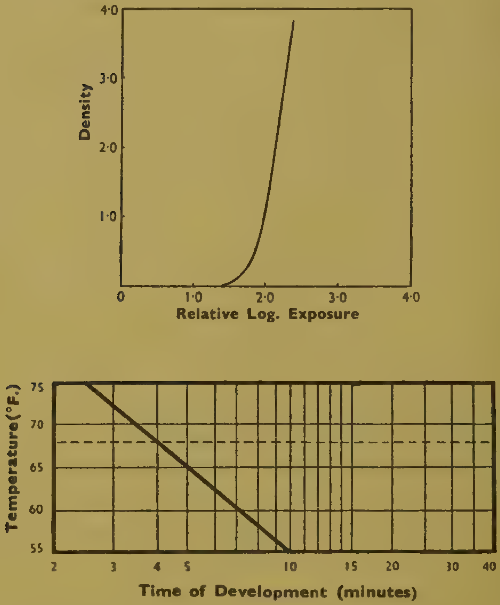
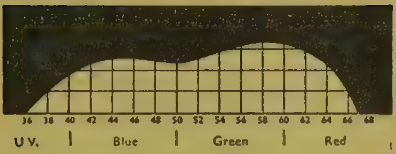


Fig. 35. Characteristics of a slow, high-contrast, high-resolution plate (Kodak Maximum Resolution Plate).

used. The contrast varies with wavelength in the same emulsion, γ-variations with more than three times being not infrequent.

The circumstances are further complicated by the fact that the characteristics of the emulsion vary in different parts of the same plate. Special attention must also be paid to the diminishing influence of a strongly exposed part of the emulsion on the density of the closely neighbouring parts. This disturbing phenomenon is alleviated if the plate is continually wiped with a soft brush during the development.



Sensitivity Curve of Tungsten Light (2848 ^{0} *K.*)

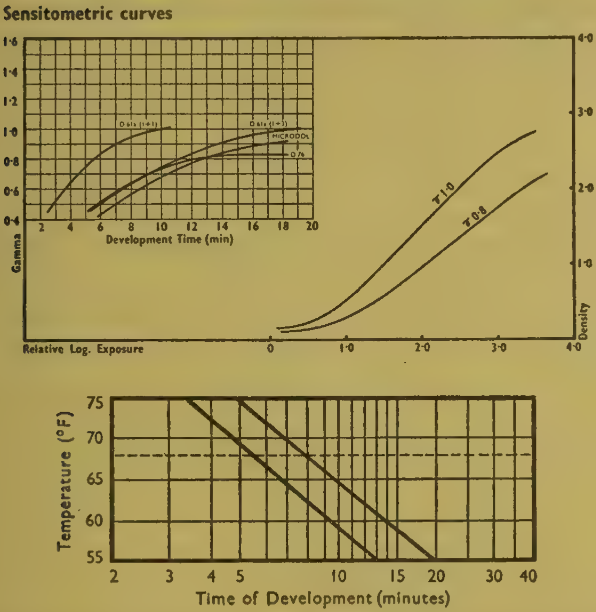


Fig. 36. Characteristics of a rapid panchromatic film (Kodak Super-XX Sheet Film).

From the presented theoretical considerations it follows that the photographic plates can only be used with high accuracy for matching two neighbouring parts with an equal density in the same plate. In photometry, sample and reference beams must thus be used. Because of the reciprocity-law failure, exposure times for both beams must be equal, and the intensity of either the sample or the reference beam must be varied continuously or discontinuously. By means of this method, which is analogous to the visual method of photometry, light intensities can, under favourable conditions, be measured with an error of 0.01 per cent. Usually the error is considerably larger.

If high accuracy is not required, it is more convenient to prepare γ-curves for the wavelength to be used and expose for the same length of time in the actual measurement. To obtain the light intensity, the density of the plate is then compared with the γ-curve. If this method is used, the accuracy is limited by variations in the response of the emulsion. Therefore, several γ-curves must be prepared from several parts of several plates, and the errors in different parts of the average γ-curve calculated. Reciprocity-law failure does not contribute to the error in this method.

In the third photographic technique it is assumed that the reciprocity law holds true. Constant intensity of light is used and the exposure time is varied continuously or discontinuously. By projecting an image of a homogeneous tissue component on the spectrographic slit and by moving the photographic plate with the aid of an evenly rotating logarithmic cam, one can rapidly produce a spectrogram, in which the height of the blackened areas at each wavelength indicates the light intensity at this wavelength. Absorbing areas are accordingly shown as depressions of the curve. A control spectrum in which the light is directed through the same slide and cover slip, presumably through a non-absorbing area in the section must naturally be also done. This method, although least accurate of the photographic techniques, has the advantage that it is more capable of resolving fine structure of absorption spectra than any other photographic or photoelectric method (see page 111).

Evaluation of the density in the photographic plate. In all photographic methods estimation of the density in different parts of the photographic plate is the endpoint of the measurement. Visual, photoelectric and grain-counting methods of measurement can be used. Visually, the density can be measured along the same lines as has been described in the chapter concerning visual measurement of light intensities. Monochromacy of the light is not of particular importance. Light scattering, which always occurs in the photographic plate, must, on the other hand, be considered. Thus, either the intensity of light is measured with a low aperture or care is taken to collect all the diffracted light with the aid of high aperture systems. A third alternative is to place one or two diffusing glass plates below the plate. In this case the intensity distribution of the light emerging from each point of the plate is practically even. In the calibration, the same method must be used as in the actual measurement.

This applies equally to the photoelectric method. For further details the reader is referred to the next section.

The number of silver particles per unit of area is an accurate index of density of a photographic emulsion. For counting methods see chapter VII. This method can only be applied when the density is low, but at very low densities it is more accurate than visual or photoelectric methods.

Photoelectric Methods.

a) *Barrier-layer cells.* In barrier-layer cells, a thin semiconducting layer of cuprous oxide or selenium on a copper plate reacts to light rays by creating a voltage between the layer and the plate. If the semiconducting layer is covered by a transparent thin layer of conducting material, the electrical changes can be measured. Either the current flowing through the photocell or the voltage across the cell is measured.

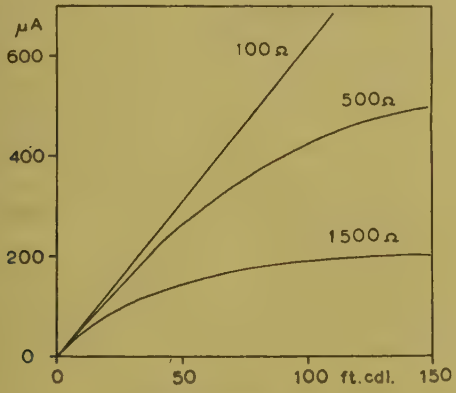


Fig. 37. Current produced at different external resistances by a barrier-layer cell as a function of the light intensity. To obtain a linear response a galvanometer with low coil resistance must be used.

In the first case (Fig. 37) the relationship between the light intensity and the electric current produced is dependent on the resistance of the external circuit. At low values of external resistance the electric current is proportional to the light intensity up to high intensities. If the external resistance is high, the increase in the current becomes progressively smaller with increasing absolute light intensity.

If no current is flowing through the barrier-layer cell, the voltage across the cell is proportional to the logarithm of the light intensity. As direct-reading electrostatic meters of sufficient sensitivity are expensive, a compensating circuit such as that shown in Fig. 38 can be recommended. The potentiometer is adjusted so that no current flows through the zero-indicator. If a galvanometer is used as a zero instrument, the voltage can be read from the galvanometer scale by short-circuiting the photocell. Reading of the potentiometer can be used as well. In this case, it is advantageous to adjust photocurrent produced by the reference beam to zero by another potentiometer R_1 while the slide wire potentiometer R_2 is fully open (Fig. 39). The light is then allowed to pass through the absorbing part of the section and R_2 is adjusted to set the zero. a is then in linear proportion to the extinction, and, when Beer's law is valid, to the concentration of the absorbing substance.

Fig. 40 shows a balanced circuit designed by *Wilcox* (1934). The use of two cells makes it possible to avoid fluctuations caused by intensity changes in the light source. Photocell 1 is used for measuring the intensity of light after the beam has passed the blank or the sample; photocell 2 is used for compensation. The blank is first introduced between the light source and cell 1. The slide-wire resistor R_2 is fully opened and resistor R_1 is adjusted until no current is flowing through the zero instrument. The blank is replaced by the sample and the balance is re-established with R_2. Per cent transmittance is then directly indicated on the slide wire. Fluctuations due to source variations are at minimum at this setting of the circuit.

In a frequently used compensating circuit with two cells simply connected opposite to each other (Fig. 41) errors due to light fluctuations are compensated only when equal intensities of light are falling on both cells. This necessitates the use of optical methods of compensation, e.g. by means of a variable aperture in front of the compensating cell or of an arrangement utilizing the inverse square

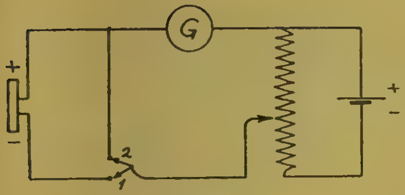


Fig. 38.

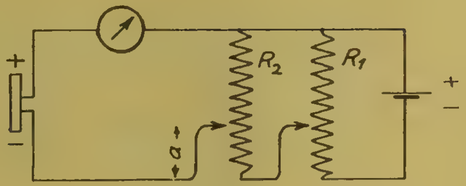


Fig. 39.

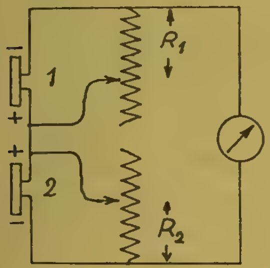


Fig. 40.

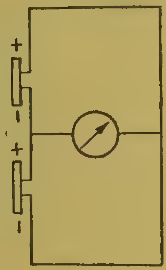


Fig. 41.

Fig. 38. A simple circuit using a barrier-layer cell. The galvanometer is adjusted with the resistance to read zero at the position 1 of the switch. Then no current flows through the cell and the voltage, which can be read from the galvanometer the switch in position 2, is directly proportional to the logarithm of the light intensity.

Fig. 39. With this circuit the extinction can be directly obtained. The photocurrent caused by the reference beam is first compensated by moving the potentiometer R_1 until the galvanometer reads zero. The sample is introduced and the extinction is then directly proportional to the adjustment a of the slide wire R_2 causing a zero reading in the galvanometer.

Fig. 40. A circuit compensating for fluctuations in the intensity of light. 1 is the measuring cell, 2 is the balancing cell. Light through the blank is first allowed to fall on cell 1, and the current is balanced with potentiometer R_1 when the slide wire R_2 reads 100. The sample beam is then measured in the cell 1 by balancing with R_2. The per cent transmission is then directly indicated on the slide wire.

Fig. 41. Two photocells simply connected against each other. Compensation is provided for intensity fluctuations when the light intensities falling on both cells are equal.

law. Setting of the compensation must be provided with a scale from which the light intensity can be read.

Sensitivity of average-sized barrier-layer cells is fairly low and electronic amplification of the currents produced by the barrier-layer cell is difficult. If the sensitivity is inadequate, it is advisable to use a vacuum phototube with an amplifier, or a photomultiplier tube. The usefulness of barrier-layer cells in microscopic photometry is normally limited to measurements in relatively large areas of tissue sections or to cases where high intensities of light can be used.

b) *Vacuum phototubes.* Vacuum phototubes have two electrodes enclosed in an evacuated glass or quartz tube. Light falling on the light-sensitive cathode induces emission of electrons from it. If voltage is applied between the cathode and the anode, movement of electrones from the negatively charged cathode to the anode follows. At constant voltage this photocurrent is dependent on the illumination of the cathode. The interdependence of the photocurrent, the applied voltage, and the intensity of light are exemplified in Fig. 42. It can be seen that with constant intensity of light the photocurrent first increases sharply with increasing voltage. When a certain current is reached, further increase in anode voltage has practically no influence on the current. The magnitude of this saturation current is dependent on the intensity of light. A linear relationship exists between the light intensity and the saturation current.

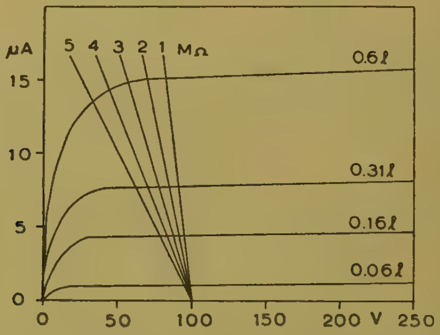


Fig. 42. Anode current as a function of anode voltage at different illuminations in a *Mullard* vacuum phototube. At an illumination of 0.31 lumen the saturation current (ca. 7 μA) is reached with an anode voltage of ca. 50 V. A load resistor of 5 MΩ would then cause a voltage drop of ca. $7 \times 5 = 35$ V. Therefore, a voltage higher than $50 + 35 = 85$ V is necessary for saturated working. The voltage drop caused by different load resistances at different currents is indicated by straight lines.

The resistance of the external load must be properly chosen. If the maximum current of the phototube (1 to 20 μA) is exceeded, deterioration of the photosensitive surface follows. As the maximum anode voltage is usually about 100 V, safety is attained with an external load resistance of 10 to 0.5 MΩ, depending on the maximum current. Too high load resistances limit the photocurrent below the saturation value, in which case the response to light will not be linear. The voltage drop caused by the load resistance is indicated by straight lines in Fig. 42. It can be seen that a saturation current of 7 μA can safely be reached with a load resistance of 5 MΩ while photocurrents of more than 15 μA cause a drop in the anode voltage below the value necessary for saturated working.

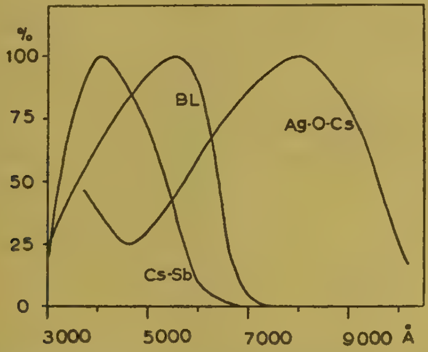


Fig. 43. Relative spectral sensitivities of a barrier-layer cell, BL; of a caesium-antimony photocathode, Cs-Sb; and of a photocathode of caesium on oxidized silver, Ag-O-Cs.

Even if no light is falling on the cathode, a certain current flows through the tube when a voltage is applied between the electrodes. This «dark current» increases with increasing voltage. It is therefore desirable to use the minimum voltage capable of maintaining the saturation current.

The spectral response of vacuum phototubes is dependent on the cathode surface. Fig. 43 shows the spectral sensitivities of a caesium-silver cathode and of a caesium-antimony cathode, as well as that of a barrier-layer cell. It will be noted that the sensitivity of the caesium-silver cathode is fairly high in infrared. This necessitates effective filtration of infrared light when measurements are done at other regions of the spectrum.

The average sensitivity of vacuum phototubes is lower than that of barrier-layer cells. It can nevertheless be conveniently increased by electronic amplification.

Fig. 44 shows the fundamental circuit of a single-tube DC amplifier. The photocurrent flows through a resistance R_g. The resulting voltage drop influences the grid voltage. This consequently increases the plate current which is indicated by a galvanometer. A simple and sensitive circuit is shown in Fig. 45.

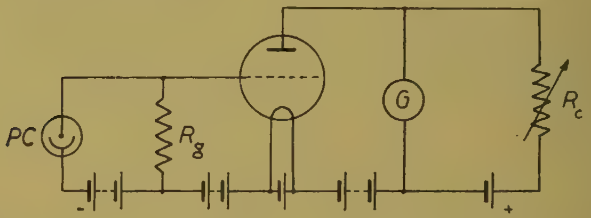


Fig. 44. Basic circuit of a DC photocurrent amplifier. The phototube, PC, causes when illuminated a photocurrent through the load resistor R_g, and the grid potential of the triode is altered. This change causes a change in the plate current which is measured with the galvanometer, G. The "dark current" is compensated with R_c when no light is falling on the phototube.

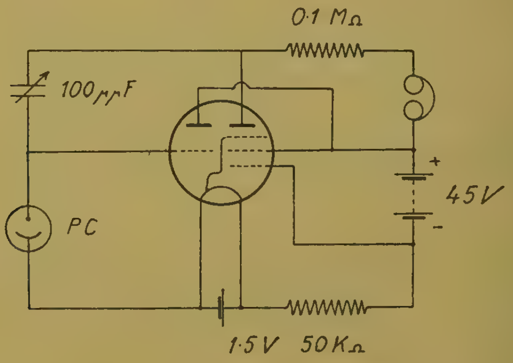


Fig. 45. An amplifier circuit designed by *Roberts* (1940). The phototube functions as a grid leak and the light intensity influences the frequency of clicks heard in a loudspeaker.

c) *Photomultiplier tubes.* Fig. 46 illustrates the structure of a typical photomultiplier tube. Light falling on the cathode causes a release of photoelectrons in the same way as in an ordinary vacuum phototube. By means of an electrostatic field these electrons are directed towards a secondary electrode, so-called dynode. Each electron, the speed of which varies according to the voltage between the cathode and the dynode, releases several secondary electrons from the dynode surface. The original photocurrent is thus magnified by the number of secondary electrons per one photoelectron.

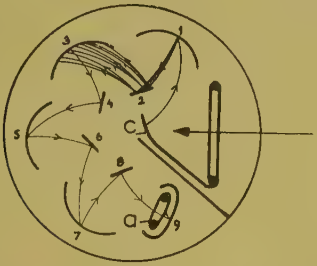


Fig. 46. Schematic drawing illustrating the structure of a photomultiplier tube. Light is indicated by the long arrow; c, photosensitive cathode; 1-9, the dynodes; a, the anode.

The secondary electrons are further directed to a second dynode, where the process is repeated. The final multiplication factor is S^n, where S is the number of secondary electrons released by each electron and n the number of dynodes. From the last dynode the electrons are collected to anode.

In RCA photomultiplier tube 931-A, which is much used in photometry, the number of dynodes is 9. By varying the voltage over each dynode stage from 25 to 100 V the current can be amplified from 100 to 1,000,000 times. The photocurrent is measured between the last dynode and the anode. The saturation current is reached at about 50 V between the last dynode and anode. The average anode current must be kept below ca. 1 mA. This saturation current is given by a light flux of ca. 0.000,1 lumen at 100 V between each dynode stage. This illustrates the extreme sensitivity of this tube.

Characteristics of individual photomultiplier tubes vary within large limits. This also concerns the dark current, which may prove troublesomely high. The voltage between the last dynode and the anode should not be increased over the value necessary for the operation at a point just giving anode-current saturation. Therefore, the circuit must be planned so that this voltage can be regulated. At low intensities of light the saturation current is reached at lower voltages than at high intensities. If a fixed dynode-anode voltage is used the maximum operation stability is not achieved at low light intensities because of an unduly high dynode-anode voltage, which increases the dark current but does not improve the sensitivity.

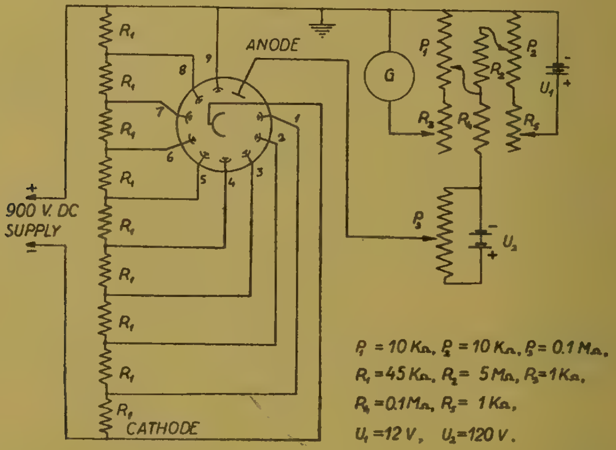

R_1
ANODE
9
8
7
6
5
4
3
2
1
G
P_1
P_2
R_2
U_1
R_3
R_4
R_5
+
900 V. DC
SUPPLY
−
P_3
U_2
CATHODE
$P_1 = 10\ K\Omega$, $P_2 = 10\ K\Omega$, $P_3 = 0.1\ M\Omega$,
$R_1 = 45\ K\Omega$, $R_2 = 5\ M\Omega$, $R_3 = 1\ K\Omega$,
$R_4 = 0.1\ M\Omega$, $R_5 = 1\ K\Omega$,
$U_1 = 12\ V$, $U_2 = 120\ V$.

Fig. 47. A photomultiplier circuit used by the writer. The voltage between the last dynode and the anode can be adjusted with potentiometer P_3. The galvanometer sensitivity can be regulated with resistor R_3 and the potentiometer P_1. Resistors P_2 and R_5 serve for compensating the dark current. The resistor R_4 limits the tube current below the dangerous limit.

Fig. 47 shows a photomultiplier circuit used by the writer in which a ready war-surplus photomultiplier unit has been used. The voltage for the dynode stages was fed from mains through an electronic stabilizer but it can equally well be supplied by a series of dry batteries. In practice, the current is kept so low that the dry batteries will keep as long as without any load.

CHAPTER XI

Statistical Analysis of Results

General Principles.

The procedure of interpreting quantitative data must be so designed that the uncertainty introduced by sampling is taken into account. For example, if two means, say from a control group and an experimental group, have been observed, the investigator is interested in the difference between the means. If any numerical difference is observed, it evidently may be

a) either due to mere chance variations, which means that there is no real difference,

b) or due to a real difference between the groups.

The investigator never *knows* with certainty which of these alternatives is true. Yet the data may enable him to draw the conclusion that one of the alternatives seems more plausible than the other. To this end, *testing a statistical hypothesis* is done.

There are certain rules to be followed in the test procedure. The first step is to define the *hypothesis to be tested*. Usually the alternative a) is taken as the hypothesis to be tested, also known as *null hypothesis*. The *alternative hypothesis*, obviously, is given in b) above.

In the testing procedure, the null hypothesis is tentatively accepted as true. Then, a *test variable* is calculated from the data, the variable being so constructed that its statistical behaviour is known in advance (i.e. that its distribution is known). As this is done, it can readily be seen whether the value of the test variable observed corresponds to its pre-calculated distribution. If the observed value is so large that, in view of the distribution of the variable, the occurrence of such a value is unlikely (this meaning some small probability, conventionally 0.05, 0.01 or 0.001), the *plausible* conclusion is drawn that the observed value is not from the pre-calculated distribution. But the distribution used in testing is always calculated presuming the correctness of the null hypothesis, whence the above leads to rejection of the null hypothesis. Correspondingly, a relatively small value of the test variable is to be taken to be in favour of the null hypothesis.

In the example above, one could calculate the difference between the means and use it as the test variable. If the difference is large, the null hypothesis will be rejected, i.e. the difference is taken as a statistical evidence of the correctness of the alternative hypothesis. The crucial point is how to determine when the observed difference is "too large". This can be done after an investigation of the variability of the observations, and the result—known as the *t*-test—will be shortly presented.

Now following the *practical rule of behaviour* outlined above the investigator may result in error. He may

a) "find" a difference where no difference exists, i.e. reject the null hypothesis even if it is correct (error of the first type),

b) not detect a real difference, i.e. accept the null hypothesis even if it is incorrect (error of the second type).

These errors are *always* present in scientific work, so that the investigator must take the risk of them. Now the very superiority of applying statistical methods lies in that *the magnitude of these risks can be controlled.* In fact, the investigator himself may choose the risk he is willing to run. Usually he chooses the risk of an error of the first type, this chosen risk being known als the *level of significance*, commonly denoted by P, and conventionally taken 0.05, 0.01 or 0.001. All the statistical test procedures in current use, on the other hand, have been so designed as to give minimum risk of an error of the second type with given P, so that the risks in drawing conclusions really are under control.

If the difference between two means is observed to be so large that in at most 5 cases out of 100 such a difference would have resulted by chance only (this statement being based on the distribution of the difference on the null hypothesis), the investigator has good reason to reject the null hypothesis as incorrect. In this case it is said that the difference is *significant* at $P = 0.05$ level, and the odds are at most 5 in 100 that the conclusion of the investigator is erroneous.

The test variable can be calculated in many ways. Some of the possible variables are more efficient than the others, being thus chosen in common use. The most common of the test variables will be presented below, together with an illustrative example. The reader is advised to follow the calculations with actual re-calculation, for otherwise he may drop some important details.

Comparison of Two Averages. The *t*-test.

We define a test variable t as follows: any random variable which is distributed normally with mean O and standard deviation σ, divided by an estimate s of σ, the estimate being calculated from the same data as the random variable with f degrees of freedom, is distributed according to the t-distribution (also known as "*Student*'s" distribution). This distribution has been tabulated for various degrees of freedom (*Hald*, 1952; *Fisher* and *Yates*, 1953) and the tables will be used in actual testing.

In comparing two averages $\bar{x}_1$ and $\bar{x}_2$ we start from the fact that the averages tend to be normally distributed. But so also does their difference $\bar{x}_1 - \bar{x}_2$, which, by the null hypothesis, is expected to equal to zero. Hence, this difference can be taken in the numerator described above. In the denominator, the estimated standard deviation of this difference will be taken.

Generally it is advisable to use a variance estimate obtained by pooling the variances of the two groups, thus:

$$s^2 = \frac{Q_1 + Q_2}{n_1 + n_2 - 2}, \tag{52}$$

where the Q's are given by formula (3), page 19. The denominator then is calculated as the square root of

$$s^2(\bar{x}_1 - \bar{x}_2) = s^2 \frac{n_1 + n_2}{n_1 n_2}, \tag{53}$$

whence

$$t = \frac{\bar{x}_1 - \bar{x}_2}{s(\bar{x}_1 - \bar{x}_2)}. \tag{54}$$

What remains is to enter the t-tables with the value calculated and with $f = n_1 + n_2 - 2$ degrees of freedom. On the row determined by f find a value next to that observed but smaller (or equal). Having found this, the P-value is found at the head (or, in some tables, at the bottom) of the column.

The pooling method above is not justified if the random errors in the two averages are not considered to be from the same source. In such cases it is preferable to calculate the variances of the averages separately by the formula (5), page 20. The sum of these estimates is then the adequate estimate of the variance of the difference between the averages. However, additional complications arise in case of unequal variances (see *Fisher*, 1950).

As an example, let us analyse an experiment. A group of 6 animals has been injected with ACTH, and a similar control group with the same volume of isotonic saline. It is wanted to know whether ACTH influences the volume of the adrenal medulla. The medullary volumes in each animal are given below together with calculations.

Animal No.	Controls x_0	x_0^2	ACTH x_1	x_1^2
1	1.9	3.61	1.0	1.00
2	2.0	4.00	1.1	1.21
3	2.2	4.84	1.9	3.61
4	2.1	4.41	1.4	1.96
5	2.0	4.00	1.8	3.24
6	2.4	5.76	1.5	2.25
$\sum x$	12.6		8.7	
$\bar{x} = \frac{(\sum x)}{n}$	2.10		1.45	
$\sum x^2$		26.62		13.27
$S = \frac{(\sum x)^2}{n}$		26.46		12.62
$Q = \sum x^2 - S$		0.16		0.65

These calculations are standard necessities in many applications. Now we want to know whether the difference of the means $\bar{x}_0 - \bar{x}_1 = 2.10 - 1.45 = 0.65$ is an indication of the effect of ACTH treatment or due to chance only.

The sums of squares are first pooled, $Q_0 + Q_1 = 0.16 + 0.65 = 0.81$. The number of degrees of freedom is $n_0 + n_1 - 2 = 6 + 6 - 2 = 10$. Pooled variance estimate is then $\frac{Q_0 + Q_1}{n_0 + n_1 - 2} = \frac{0.81}{10} = 0.0810$.

This multiplied by $\frac{n_0 + n_1}{n_0 n_1} = \frac{12}{36} = \frac{1}{3}$ gives the variance of the difference of the means $s^2(\bar{x}_0 - \bar{x}_1) = 0.0270$, the square root of which is the standard deviation of the difference of the means $s(\bar{x}_0 - \bar{x}_1) = 0.16$. Now

$$t = \frac{\bar{x}_0 - \bar{x}_1}{s(\bar{x}_0 - \bar{x}_1)} = \frac{0.65}{0.16} = 4.1\,.$$

From the table of *t*-distribution it is seen that with 10 degrees of freedom a value of $t = 3.17$ corresponds to a probability 1 out of 100 that the difference of means is due to mere chance ($P = 0.01$). As t is in the present experiment larger than 3.17, the probability of the observed difference being due to chance is less than 1/100 ($P < 0.01$). The *t*-test thus favours the hypothesis that ACTH causes a decrease in the volume of the adrenal medulla.

In some applications it is wished only to test whether an observed mean differs from zero. This test is performed completely in analogy with the above, simply by

$$t = \frac{\bar{x}\, n}{s(x)}\,, \tag{55}$$

where $s(x)$ is given by formula (4), page 20. There are $n - 1$ degrees of freedom.

The use of the *t*-test is by no means limited to two averages. As it is seen from the general definition given at the beginning of this section, any random variable with normal distribution can be concerned. For applications, see standard texts, e.g. *Fisher* (1950). However, because of frequent misuses, the correct test for the correlation coefficient is given. In correlation problems, the *t*-variable should be calculated as the square root of

$$t^2 = \frac{(n-2)\, r^2}{1 - r^2}\,, \tag{56}$$

there being $f = n - 2$ degrees of freedom. Any attempt to calculate the standard deviation of the correlation coefficient should be avoided, for these calculations have sense only if

a) the number of observations, n, is very large.

b) the true value of the correlation coefficient is very small or equal to zero;

these conditions rarely holding. The formula (56) is always correct, the test being whether the observed coefficient differs significantly from zero.

The sampling distribution of the correlation coefficient is far from normal, hence the above warning. Only if the true correlation equals to zero, the sampling distribution of r tends to normal for large number of observations in the sample.

More than Two Averages. Analysis of Variance.

Often it is desired to analyse simultaneously more than two groups in order to verify whether any difference between the group averages exists. In such cases *analysis of variance* will usually be a suitable method of analysis. The main principle in analysis of variance is that the total variability in the observation material—measured by the sum of squares—is broken down according to the different sources of variability. Having obtained the observations the investigator readily can calculate the total sum of squares. Evidently, the magnitude of this quantity depends on both the variability *between groups* and the variability *within groups*. A close investigation of the effect of the different sources of variability may result in conclusions concerning the significance of the observed differences. Only the general case will be presented here, and the reader is referred to special texts for all details, e.g. *Snedecor* (1946) and *Fisher* (1950).

The first step is to calculate the total sum of squares over the entire material (i.e. with no regard to the fact that some of the groups may be control, the others experimental groups), by the general formula (3), page 19. Then, this quantity is broken down into two parts, viz.

a) $Q_B =$ sum of squares due to the differences *between* groups,

b) $Q_0 =$ *residual* sum of squares, the magnitude of which depends on the variability *within* groups.

If the material consists of $n = \sum n_i$ observations, there are altogether $n - 1$ degrees of freedom. Now if there are k (say 3) groups to be compared, it is said that $k - 1$ (say 2) degrees of freedom correspond to the differences between groups. Therefore $(n - 1) - (k - 1)$ degrees of freedom correspond to the residual variation. Any splitting of sums of squares is corresponded by a splitting of the number of degrees of freedom.

Now any sum of squares divided by the number of degrees of freedom gives a variance estimate, wherefore variances can be calculated for both "between"- and "within"-variation on the basis of the respective sums of squares and degrees of freedom. If the null hypothesis (that there is no essential difference between groups) holds, then, as shown in mathematical statistics, these estimates will, in the average, be equal. So the proportions of their magnitudes can be used as a basis for deciding whether any difference between groups exists.

In practice, the most straightforward way of calculations is as follows: First, the over-all sum of squares Q is calculated. By the way, the general subtraction term is obtained (this being the square of the over-all total divided by n). Then, group subtraction terms are calculated (group total squared and divided by the number of observations n_i in the group). Now, the sum of the group subtraction terms less the general subtraction term equals to the sum of squares "between" groups, thus:

$$Q_B = \sum \frac{(\sum x_i)^2}{n_i} - \frac{(\sum\sum x_i)^2}{n}. \tag{57}$$

The residual sum of squares is obtained by subtraction:

$$Q_0 = Q - Q_B. \tag{58}$$

It is customary to arrange the data in a table as follows:

Source of variation	Sum of squares	Degrees of freedom	Variance	v^2	P
Between groups . .	Q_B	f_B	$s_B^2 = \frac{Q_B}{f_B}$	$\frac{s_B^2}{s_0^2}$	.
Within groups . . .	Q_0	f_0	$s_0^2 = \frac{Q_0}{f_0}$	.	.
Total	Q $= Q_B + Q_0$	$n - 1$ $= f_B + f_0$	.	.	.

The degrees of freedom for each row were given above. The variance column is obtained by dividing the Q's by the f's. Finally, the comparison between the effects of the two sources of variation is performed by calculating the quantity

$$v^2 = \frac{s_B^2}{s_0^2}, \tag{59}$$

known as *variance ratio*, also denoted by F in honour to *Fisher*. If there are no essential differences between the groups, v^2 will remain small, somewhere in the neighbourhood of 1, but the more there are differences, the larger will v^2 be. In fact, v^2 is another *test variable* whose distribution is known and tabulated, wherefore the significance of any observed v^2 can be determined by means of tables, e.g. *Hald* (1952); *Fisher* and *Yates* (1953), essentially in a similar way as was explained in connection with the t-test. The only difference is that now we have to enter the table with two numbers of degrees of freedom, viz. f_B and f_0 (denoted by f_1 and f_2 or n_1 and n_2 in the tables). If s_0^2 is larger than s_B^2, the calculation of v^2 should be made by interchanging the two quantities so that v^2 always is at least equal to unity. In this case, f_B and f_0 must be interchanged also.

Consider now an experiment in which three independent groups were subjected to different treatments. Group 0 was injected with saline, group 1 with ACTH and group 2 with nicotine. The difference between groups 0 and 1 was above tested with the t-test. Now analysis of variance is applied to study the effect of the treatments on the volume of the adrenal medulla in the three groups. First, the same calculations are performed as in the above example.

Animal No.	x_0	x_1	x_2	Total
1	1.9	1.0	3.9	
2	2.0	1.1	4.1	
3	2.2	1.9	3.9	
4	2.1	1.4	3.4	
5	2.0	1.8	3.7	
6	2.4	1.5	4.5	
$\sum x$	12.6	8.7	23.5	44.8 $(= \sum\sum x)$
$\bar{x}$	2.10	1.45	3.92	
$\sum x^2$	26.62	13.27	92.73	132.62 $(= \sum\sum x^2)$
S	26.46	12.62	92.04	111.50 $\left(= \frac{(\sum\sum x)^2}{\sum n_i}\right)$
Q	0.16	0.65	0.69	21.12

If all the three means are to be compared, analysis of variance will be applied. First calculate an overall or total sum of squares as if all the groups were combined into one, larger group. This calculation is shown in the table above, $Q = 21.12$. This sum of squares is broken down into two parts according to the source of variation, as told on page 139:

$Q_B = 26.46 + 12.62 + 92.04 - 111.50 = 19.62$,

$Q_o = 21.12 - 19.62 = 1.50$ (check: this must equal to the sum of the group Q's, $0.16 + 0.65 + 0.69 = 1.50$).

There are $(n_0 + n_1 + n_2) - 1 = 17$ degrees of freedom altogether, of which 2 (as there are 3 groups) are due to differences between groups. Enter the figures in the analysis table. Complete the calculations.

Source of variation	Q	f	s^2	v^2	P
Between groups	19.62	2	9.81	98.1	0.001
Within groups	1.50	15	0.10	.	.
Total	21.12	17	.	.	.

The table of v^2-distribution shows that the chances are by far less than 1 in 1000 that this v^2 would have been so large at random only, $P < 0.001$. Thus, real differences exist.

However, in this test the question remains unanswered what are the differences between groups that matter. For complete analysis, see standard texts (*Snedecor*, 1946; *Fisher*, 1950). Here the example is continued by splitting Q_B for each single degree of freedom. This can be done in several ways, one of which is a) control *contra* treatments, b) ACTH *contra* nicotine.

First, pool the groups 1 and 2 as if they were one single group, and calculate the subtraction term for this pooled group. Denoting the subtraction term by S, we obtain $S_{12} = \frac{(8.7 + 23.5)^2}{12} = 86.40$ (the subscript 12 meaning the pooled group). Now the part-sum of squares due to the difference control *contra* treatments is obtained by $S_0 + S_{12} - S$ (control subtr. term plus pooled subtr. term less general subtr. term) $= 26.46 + 86.40 - 111.50 = 1.36$. This reduced from the total between-sum of squares ($Q_B = 19.62$) gives the part-sum of squares due to the difference between groups 1 and 2. So an analysis table can be constructed.

Source of variation	Q	f	s^2	v^2	P
Control *contra* treatments	1.36	1	1.36	13.6	0.005
Between ACTH and nicotine . .	18.26	1	18.26	182.6	0.001
Total between groups	19.62	2	9.81	98.1	0.001

The total row is taken from the analysis table above. The v^2's have all been calculated by dividing each variance by the within-groups variance (known also as residual variance). From the above it is seen that both comparisons show significant effects of treatments.

In *correlation* and *regression* problems, analysis of variance can be applied to show the explaining capacity of the regression equation used. The calculation is quite simple, for

$$Q_B = Q_R = r^2 Q, \tag{60}$$

where we write Q_R rather than Q_B for the regression part of the sum of squares. For this part, there is one degree of freedom, $n - 2$ remaining for the residual sum of squares. The reader may by some calculation verify that v^2 in this case reduces to the square of the t-variable already given in formula (56), page 137.

Let us examine the appropriate method with the aid of an example. There are many independent organs such as the adrenal medulla which cannot be weighed as they are enclosed in another organ. Therefore, an estimate of weight must be obtained by some other means, e.g. by area measurements in serial sections. To study the adequacy of that kind of estimate, adrenal glands were weighed and cut in a complete series of sections. The area of each section was measured planimetrically, and the volume of each gland was calculated by multiplying the sum of areas with the section thickness.

It is desired to obtain an idea how well the volume predicts the weight. A study of the scatter diagram (Fig. 48) reveals much, but often calculations are needed. If so, it can be assumed that weight y is a linear function of volume x so that a weight estimate (regression estimate) Y can be calculated by means of the regression equation

$$Y = a + bx, \tag{61}$$

where the constants a and b are to be estimated from the data. The estimation formulas are simple:

$$a = \bar{y} - b\bar{x}, \tag{62}$$

$$b = \frac{T_{xy}}{Q_x} = \frac{r s(y)}{s(x)}, \tag{63}$$

where the symbols are given earlier [see formulas (1), p. 18; (3), p. 19; (5), p. 20; (14), p. 25; (16), p. 25].

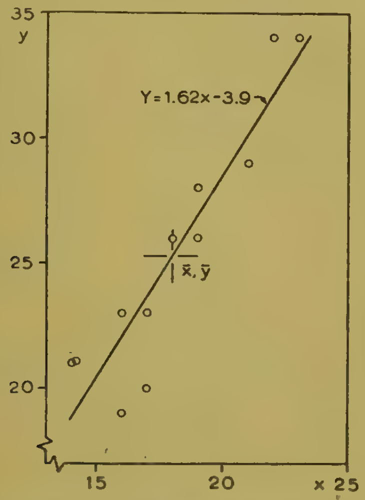


Fig. 48. Scatter diagram showing relations between adrenal volume *(x)* and weight *(y)*.

If now, using equation (61) an estimate of weight is calculated for each gland, it can be seen that the estimation and the actual weights differ: there exists some *residual variation*, i.e. the regression equation is not capable of totally explaining the original variability in the weights, in y. If the residual variation is large, the regression evidently is poor if of any practical value, whence a study of variation is necessary. It is performed in analogy with analysis of variance, applying the formula (60) in calculating the regression sum of squares. If the variance estimate based on this sum of squares is large as compared with the residual variance, then the regression explains much of the variability in y.

The only addition to computations already learned as standard is to calculate the products xy for each animal. The arrangement of computations is shown at the bottom of the table.

Animal No.	Volume cu.mm. x	Weight mg. y	Regression estimates Y
1	17	20	23.6
2	19	26	26.9
3	16	23	22.0
4	22	34	31.7
5	21	29	30.1
6	19	28	26.9
7	16	19	22.0
8	18	26	25.3
9	14	21	18.8
10	17	23	23.6
11	14	21	18.8
12	23	34	33.4
	216	304	Sum
	18.0	25.3	Mean
	3982	7990	Sum of squared observations
	3888	7701	Subtraction term
	94	289	Sum of squares
	8.55	26.27	Variance
	2.9	5.1	Standard deviation
	5624		Sum of products $\sum xy$
	5472		Subtraction term $\frac{\sum x \sum y}{n}$
	152		Product-sum $T_{xy} = \sum xy - \frac{\sum x \sum y}{n}$

$$b = \frac{152}{94} = 1.62,$$

$$a = 25.3 - 1.62 \cdot 18.0 = -3.9.$$

Regression equation:

$Y = 1.62x - 3.9$. Regression estimates calculated from this equation are seen in the table.

Correlation coefficient:

$$r = \frac{152}{\sqrt{94 \cdot 289}} = 0.92,$$

$$r^2 = 0.8505.$$

Regression sum of squares:

$$Q_R = r^2 Q_y = 0.8505 \cdot 289 = 246.$$

Residual sum of squares:

$$Q_0 = Q_y - Q_R = 289 - 246 = 43.$$

Analysis of variance:

Source of variation	Q	f	s^2	v^2	P
Regression	246	1	246	57.2	0.001
Residual	43	10	4.3	.	.
Total	289	11	.	.	.

The analysis of variance shows that the regression is highly significant, P being smaller than 0.001. This shows that there is a true dependence between the measured volume and the weight of the glands. However, this fact does not imply that the measured volume is a good indicator of weight. The value of the volume as an estimate of weight can nevertheless be assessed with the aid of the above data.

One way of doing this is to compare the original standard deviation of weight $s(y) = \sqrt{\frac{289}{11}} = 5.1$ with the residual standard deviation left after the regression equation has been fitted $= \sqrt{4.3} = 2.1$. The latter is about 40 % of the former, i.e. 60 % of the original variation is explained by the regression equation, which is not too bad.

Another way, frequently useful in practice, is to calculate the confidence limits of the weight estimate obtained from the volume. With 10 degrees of freedom, a value of $t = 2.23$ corresponds to the level $P = 0.05$. By multiplying the residual standard deviation with this value the confidence limits $\pm 2.23 \cdot 2.1 = \pm 4.7$ mg. are obtained. This means that in 95 % of cases a weight estimate calculated from the regression equation is within these limits. For example, a measured volume of 21 cu.mm. indicates that in 95 % the true weight is between 25.5 and 34.9 mg.

Comparison of Distributions. χ^2-test.

In both t- and v^2-tests above the assumption of *normal distribution* is inherent. The tests, however, are valid even if there is some non-normality in the distributions concerned. Yet the normal assumption should not be extended without care into very skew or otherwise unusual distributions, and the above tests should not be used under such conditions. An important test known as χ^2-test has the advantage that it is *independent of the form of the distribution of the statistical variable concerned.* In this test, two or more observed distributions, in terms of absolute frequencies are compared. A general formula for calculating the test variable involves a series of "expected" frequencies to be compared with those actually observed, the expectation being based on either the observations or an assumption of the form of the distribution. In an example below a common method of calculating the expected frequencies on the basis of observations is shown. The general formula for comparing an empirical distribution with the "expected" frequencies is

$$\chi^2 = \sum \frac{(O - E)^2}{E}, \tag{64}$$

where O stands for the observed, E for the expected frequency. Sometimes it is necessary to reduce the number of frequency groups, for the frequency should in no case be 5 or less. Usually, the number of degrees of freedom is $k - 1$ if there are k frequency groups. If groups have been pooled, the number of degrees of freedom are correspondingly reduced. From (64) it can be seen that the larger the differences between the frequencies, the larger χ^2, whence a large observed value of χ^2 is likely to lead to rejection of the null hypothesis (according to which the observed and expected frequencies are statistically the same).

The number of degrees of freedom in χ^2-test depends not only on the number of frequency groups but also on the method of determining the expected frequencies. If these are determined on the basis of the observations only, say from the marginal totals, the number of degrees of freedom is $k - 1$ as given above. However, if additional parameters have been assumed or calculated, this figure must be correspondingly reduced (*Fisher*, 1950).

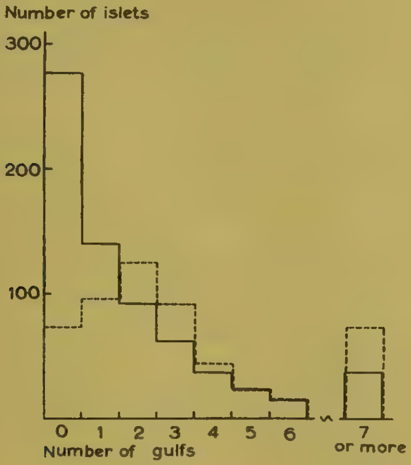


Fig. 49. Frequency distributions of gulf numbers in silver islets (solid line) and in acid-phosphatase-negative islets (broken line).

An example will make clear the practical calculations necessary. In the adrenal medulla of the rat, islets of irregular form can be demonstrated with a silver technique and with a histochemical phosphatase method. The doubt arose that the two techniques do not always demonstrate identical things, and the number of "gulfs" in each islet was counted in a gland serially cut, whose every other section had been treated with silver, every other to show acid phosphatase. The results obtained are seen in Fig. 49 and in the following table.

χ^2-example:

Number of gulfs	Number of islets			Total %	Expected fr'ies		Differences		Contributions to χ^2	
	Ag	Acid	Total		Ag	Acid	Ag	Acid	Ag	Acid
x	O_1	O_2	O_1+O_2	P	E_1	E_2	O_1-E_1	O_2-E_2	$\frac{(O_1-E_1)^2}{E_1}$	$\frac{(O_2-E_2)^2}{E_2}$
0	277	73	350	28.5	195.2	154.8	+ 81.8	− 81.8	34.3	43.2
1	140	96	236	19.2	131.6	104.4	+ 8.4	− 8.4	0.5	0.7
2	92	125	217	17.7	121.1	95.9	− 29.1	+ 29.1	7.0	8.8
3	62	92	154	12.6	85.9	68.1	− 23.9	+ 23.9	6.6	8.4
4	37	44	81	6.6	45.2	35.8	− 8.2	+ 8.2	1.5	1.9
5	24	23	47	3.8	26.2	20.8	− 2.2	+ 2.2	0.2	0.2
6	15	16	31	2.5	17.3	13.7	− 2.3	+ 2.3	0.3	0.4
7 or more	38	74	112	9.1	62.5	49.5	− 24.5	+ 24.5	9.6	12.2
Total	685	543	1228	100.0	685.0	543.0	.	.	60.0	75.8
									$\chi^2 = 135.8$	

If the islets are identical in both cases, the distributions of the number of gulfs should not differ significantly. This is the null hypothesis.

In the table the observed frequencies are shown in the columns O_1 and O_2. Adding these, row by row, the "total" column is obtained. This total distribution represents the best estimate of the "true" distribution if the null hypothesis is true, whence the test is based on this column, both observed distributions being compared with this. The next practical step is to calculate the per cent frequencies from the total column, say P. Now if the two distributions are statistically identical, their "expected" frequencies can be calculated by giving the bottom totals (685 and 543) a distribution of the P column. First multiply by 685 the P's, thus obtaining the E_1 column, and similarly for E_2. Then the differences of corresponding O and E are obtained by subtraction. Finally, each difference is squared and divided by the corresponding expected frequency, whence a series of contributions to chi-square is obtained. The sum of these is χ^2 required. In this case the number of degrees of freedom is 7.

An extremely large value $\chi^2 = 135.8$ is obtained. From a table of χ^2-distribution (*Hald*, 1952; *Fisher* and *Yates*, 1953) the probability that this were a result of chance only is seen to be by far less than

0.001, so that the two distributions can safely be regarded as different. A study of the contributions to chi-square reveals that the most important differences occur in islets with no, 2-3 and 7 or more gulfs.

If there are two frequency groups only and two such distributions are to be compared, the situation is known as *four-fold table* analysis. In this case the calculation of χ^2 is somewhat simpler, for if the four-fold table is put in the form

a	b	$a+b$
c	d	$c+d$
$a+c$	$b+d$	$a+b+c+d$

then the formula is

$$\chi^2 = \frac{(ad-bc)^2(a+b+c+d)}{(a+b)(c+d)(a+c)(b+d)} \tag{65}$$

with 1 degree of freedom.

Often the four-fold table is of use, particularly when dealing with data of "on-off" type. In an analysis concerning sampling methods in tissue sections, two neighbouring and thus very similar thin sections were taken. On both, points were thrown and the number of hits counted. However, the method for locating the points (i.e. point-sampling methods) were different, whence the question may arise whether the two methods give an essentially similar result. The data are given here:

	Section A	Section B	Total
Hits	10	18	28
Non-hits	17	11	28
Total	27	29	56

The quantity χ^2 is quickly calculated by

$$\frac{56 \times (17 \times 18 - 10 \times 11)^2}{27 \times 29 \times 28 \times 28} = 3.50.$$

The number of degrees of freedom is always 1 for four-fold tables. From the table proper it is observed that the odds are between 5 and 10 in 100 that so a large χ^2-value could have been observed by chance only (without real difference between the sections), $0.05 < P < 0.10$. Therefore, the two point sampling methods cannot, without considerable risk of error, be considered as dissimilar.

Yates' correction. In case of small frequencies it is advisable to make a correction in the formula above according to *Yates* (see *Fisher,* 1950). In the numerator of chi-square the determinant $ad - bc$ appears. If $ad > bc$, reduce both a and d by ½ and add ½ to both b and c, if $ad < bc$, reverse the action. In this example, $ad < bc$, whence the determinant should be calculated as $16\frac{1}{2} \times 17\frac{1}{2} - 10\frac{1}{2} \times 11\frac{1}{2}$. If this is done, $\chi^2 = 2.58$ is obtained, a value essentially lower than that above. The probability of exceeding this by chance only is $P > 0.1$, whence there is no reason to believe that the two sampling methods lead to different results. Note that the uncorrected chi-square gave a slightly "over-significant" result.

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